Agricultural Research Institute

PUSA

vl alcohol, 2:1 by volume

ıterial into crystalline
.ith small amounts of
ıcated by Loening and
crystalline appeara   ·n
ıter twelve addition   ex-
..   ᴏᴜ ᴜ.   ʳ yielded a deposit which by
.ımount of methyl alcohol was at once trans-
stalline product.   The purification of the phre-
ʷ .ver, not interrupted at this stage but the oper-
a   ·   repeated four times more.

Tᴜ. phrenosin was then recrystallized four times from hot
85 per cent ethyl alcohol. Seed crystals were added to the solu-
tions which were allowed to stand ҭ a temperature of 40°. Crys-
tallization always took place very readily. The use of a Dewar
flask as described by Rosenheim[5] was not necessary. This phren-
osin still contained traces of amorphous material. It must also be
said that the phrenosin of all the mother liquors of this series of
crystallizations did not become crystalline when Loening and
Thierfelder's procedure was applied to them. The yield of phren-
osin was 3 per cent of the white matter. It melted at 200° with
slight decomposition. The optical rotation was

$$[\alpha]_D^{20} = \frac{+ 0.26° \times 100}{1 \times 7.31} = + 3.6° \text{ (in pyridine)}$$

[4] Loening, H., and Thierfelder, H., *Z. physiol. Chem.*, **68**, 464 (1910).
[5] Rosenheim, O., *Biochem. J.*, **10**, 145 (1916).

V.

I

meltea .
was 399.   0.3890 gm.

The optical rotation

$$[\alpha]_D^{n} = \frac{+0}{1}$$

Oxida

acid was oxidized
ylor and Levene.[1]   ,........
the sake of complete oxidation.   The results
76–77° and solidified at 75°.

Fractionation of Ethyl Ester of Oxidized Cerebronic

The oxidized cerebronic acid was esterified and fract..nated
in the manner described by Taylor and Levene.[1]   37.3 gm. of
crude ester were used.

First Distillation— The substance distilled at 200 210° at 0.6
mm. (bath at 230°), giving 34 gm. of the esters.   There remained
a residue of 3.3 gm.

Second Distillation—The 34 gm. of esters obtained in the first
distillation were redistilled, giving 25 gm. of esters distilling at
185–195° at 0.3 mm. (bath at 215–220°) and 9 gm. of residue.

The second distillate was saponified.   The free acid was re-
crystallized five times from a mixture of low boiling petrolic ether
and acetone or from ether.   The glistening plates thus obtained
melted at 76.5–77° and solidified at 75–74°.   The molecular weight
found was 359.   0.2886 gm. substance: 8.03 cc. 0.1 N NaOH.

The substance had the following composition.

3.130 mg. substance: 8.970 mg. CO_2 and 3.520 mg. H_2O
3.725 "   "   : 10.675 "   "   " 4 380 "   "
$C_{24}H_{48}O_2$. Calculated. C 78.18, H 13.13
                   Found.   " 78.15, " 12.58
                                " 78.14, " 13.15

This acid was esterified. 11.5 gm. of the esters were obtained and refractionated.

Third Distillation – Three fractions were obtained in this case. Fraction 1 weighed 1.2 gm. It yielded an acid of molecular weight 355. 0.2932 gm. substance: 8.24 cc. 0.1 N NaOH. Fraction 2 weighed 7.7 gm. It yielded an acid of molecular weight 359. 0.4350 gm. substance: 12.13 cc. 0.1 N NaOH. Fraction 3 (residue) weighed 2.1 gm. and gave an acid of molecular weight 364. 0.4321 gm. substance: 11.88 cc. 0.1 N NaOH.

Fourth Distillation—6 gm. of the second fraction of the third distillation were redistilled. The distillate weighed 2.5 gm. It yielded an acid of molecular weight 357. 0.3160 gm. substance: 8.84 cc. 0.1 N NaOH.

Fifth Distillation- The fourth distillation and Fraction 1 of the third distillation were combined (3.7 gm.) and redistilled The distillate (175° at 0.4 mm., bath at 205°) weighed 1 gm. It yielded an acid which melted at 76° and solidified at 75.5°. Klenk's acid, $C_{23}H_{46}O_2$, melted at 78.5°. The molecular weight was 354. $C_{23}H_{46}O_2$ has the molecular weight 354.4. 0.3287 gm. substance: 9.28 cc. 0.1 N NaOH.

The acid had the following composition.

4.305 mg. substance· 12.310 mg. CO_2 and 5.010 mg. H_2O
$C_{24}H_{46}O_2$. Calculated. C 77.89, H 13 09, mol. wt. 354.4
                   Found.   " 77.97, " 13.02, "   " 354

The acids obtained from the mother liquor of crystallization of the material obtained from the second distillate were esterified. These esters were then combined with the residues of the third and fifth distillations and the combined material was redistilled. The residue from this distillation was combined with the residues of the first and second distillations and the material was saponified. From this an acid was obtained which melted at 76-78° and

solidified at 74–73°. It had a molecular weight of 378. 0.2857 gm. substance: 7.61 cc. 0.1 N NaOH.

This acid was esterified. 6 gm. of the ester were distilled. The distillate (191–194° at 0.4 mm., bath at 210–211°) yielded an acid which was crystallized from acetone. The molecular weight was 368. 0.0989 gm. substance: 2.68$_5$ cc. 0.1 N NaOH.

The composition of the substance was as follows:

4.080 mg. substance: 11.665 mg. CO_2 and 4.790 mg. H_2O

$C_{24}H_{48}O_2$. Calculated. C 78.18, H 13.13, mol. wt. 368.4

Found.   " 78 00, " 13.13, "   " 368

The residue of 4.1 gm. yielded an acid which did not crystallize from any solvent. It melted at 76–78° and solidified at 76–73°. This fraction consisted in the main of impure cerebronic acid. This substance had the molecular weight of 400. 0.1049 gm. substance: 2.62$_3$ cc. 0.1 N NaOH.

The composition of the substance was as follows:

4 055 mg. substance: 11.2?0 mg. CO_2 and 4.665 mg. H_2O

$C_{25}H_{50}O_3$. Calculated. C 75.30, H 12.65, mol wt. 398.4

Found.   " 75.38, " 12.87, "   " 400

The rotation of the substance was

$$[\alpha]_D^{25} = \frac{+ \; 0.18° \times 100}{1 \times 8} = + 2.25° \text{ (in pyridine)}$$

THE CHEMICAL COMPOSITION OF THE HUMAN FETUS*

By MAURICE H. GIVENS

(From the Northwestern Yeast Company, Chicago)

AND ICIE G. MACY

(From the Research Laboratory, Children's Fund of Michigan, Detroit)

In order to provide sufficient nourishment for both mother and fetus it is important to know how much material is laid down in the fetus at different periods of its development. This is consequential, not only for the sake of determining the requirements for fetal nutrition itself, but for appraising the nutritive drain on the maternal body at various stages of gestation. Slemons (2) says there are two ways of estimating the substances required for fetal nutrition; first, by assuming that the requirements of the fetus are similar to those of the new-born which is maintained by its mother's milk and secondly, by analyzing the body of the fetus on the supposition that the substances found therein represent its requirements for growth.

This report deals with the length, wet weight, dry weight or total solids, water, total ash, calcium, and magnesium content of twenty-five human fetuses ranging from 2 to 8 months in age. In addition, these data are summarized together with the available analyses in the literature (3-9) thus giving *in toto* the chemical composition of 96 human fetuses ranging from $1\frac{1}{2}$ to 10 lunar months in age from which growth diagrams have been constructed.

7

The fetuses used in this study were collected at the time of adverse conditions in pregnancy or labor. All specimens were preserved in either alcohol or formaldehyde with but two exceptions and they were kept on ice until the analyses were begun. The cord and all extraneous materials were removed before the body weights were taken. After the crown-heel length was measured with a metal tape each fetus was placed in a weighed porcelain crucible and brought to a constant weight in a low temperature oven. Incineration was begun over the open flame and when carbonization had taken place the ashing was completed in the muffle furnace. The modified McCrudden method (10) was used in the determination of calcium and magnesium in the ash.

It is not possible to determine fetal age with any certainty because in any particular case too little is known of the exact time relations existing between menstruation and ovulation, and between ovulation, coitus, and fertilization (11). The age of the fetus dates from the moment of fertilization to the cessation of development. In specimens obtained by operation or abortion this period may be known; in others it is not. The indefiniteness attached to the estimate of the age of the fetus necessarily vitiates the accuracy of any growth diagram that may be constructed and, consequently, any computation of the weight or length of the human fetus at various periods in intrauterine life is very inexact. The results from the analyses of embryos of various ages, however, are of distinct value since they do give a close approximation of fetal requirements.

In the twenty-five new cases reported in Table I, the ages of the fetuses have been calculated by applying the simple, modern, age-size formula of Arey (12). By this formula the age in lunar months is determined by multiplying the crown-heel length in cm. by 0.2. No corrections were made for the three fetuses falling below 10 cm. because their length fell within so close a proximity. Human fetuses of the same length may vary widely in chemical composition.

Although there are marked variations between fetuses of the same length, there is a definite relationship between age and

average composition as illustrated graphically in the semilogarithmic Chart 1 which summarizes the chemical analyses of 96 human

<div align="center">TABLE I</div>

<div align="center">*Composition of Human Fetus*</div>

Fetus No.	Sex	Crown-heel length	Age*	Weight		Ash		Calcium		Magnesium	
				Wet	Dry	Total	Dry weight	Total	Total ash	Total	Total ash
		cm.	lunar mos	gm	gm.	gm	per cent	gm	per cent	gm	per cent
13	♂	9 0	1 8	6 4	0 49	0 02	4 1	0 004	18 0	0 009	46 0
8	♂	9 5	1 9	14 0	1 47	0 15	10 2	0 041	27 1	0 004	2 7
1	♂	9 6	1 9	11 0	1 26	0 11	8 7	0 034	31 2	0 003	2 8
2	♂	10 6	2 1	19 0	2 27	0 20	8 8	0 047	23 5	0 003	1 6
10	♂	11 0	2 2	33 0	3 04	0 32	10 5	0 071	22 3	0 007	2 1
9	♂	11 5	2 3	32 0	4 40	0 60	13 6	0 162	27 0	0 033	5 6
4	♂	12 5	2 5	33 0	3 31	0 48	14 5	0 083	17 3	0 045	9 3
3	♂	13 1	2 6	131 0	15 28	1 60	10 5	0 467	29 3	0 036	2 2
15	♂	15 0	3 0	71 0	7 16	1 02	14 2	0 257	25 3	0 082	8 0
11	♂	16 0	3 2	86 0	8 90	1 02	11 4	0 243	23 9	0 058	5 7
5	♂	18 3	3 7	128 0	14 28	1 43	10 0	0 374	26 2	0 030	2 1
17	♂	20 0	4 0	201 0	16 70	1 87	11 2	0 578	30 9	0 066	3 5
12	♀	21 0	4 2	113 0	18 12	2 83	15 6	0 842	29 7	0 087	3 1
23	♂	21 0	4 2	161 0	19 52	2 66	13 6	0 787	29 6	0 057	2 4
16	♂	21 0	4 2	210 0	25 50	2 93	11 5	0 845	28 8	0 023	0 8
24	♀	22 0	4 4	172 0	26 74	3 54	13 2	1 038	29 3	0 031	0 9
25	♂	23 5	4 7	360 0	41 13	4 84	11 8	1 415	29 2	0 113	2 3
22	♀	24 0	4 8	312 0	37 73	4 92	13 0	1 485	30 2	0 077	1 6
6	♂	24 1	4 8	296 0	36 38	5 15	14 2	1 579	30 7	0 170	3 3
7	♂	28 0	5 6	433 0	54 38	8 57	15 8	2 973	34 7	0 282	3 3
21	♀	29 5	5 9	314 0	65 89	10 27	15 6	3 672	35 7	0 303	2 9
14	♀	36 5	7 3	1107 0	177 62	35 85	20 2	11 512	32 1	0 706	2 0
20	♂	36 5	7 3	1071 0	161 37	18 69	11 6	5 981	32 0	0 324	1 7
18†	♀‡	39 5	7 9	1060 0	199 0	17 59	8 8	6 253	35 5	0 670	3 8
19†	♂§	40 0	8 0	1170 0	266 5	42 64	16 0	13 703	32 1	1 443	3 4

* Arey's formula = standing height (cm.) × 0.2 = age (months).

† Twins.

‡ Born September 30, 1920, at 12.08 a.m.; died 3.15 a.m.

§ Born September 30, 1920; died October 4, 1920.

fetuses. The age in lunar months is plotted on the horizontal arithmetic scale and the weights and lengths of the fetuses on the
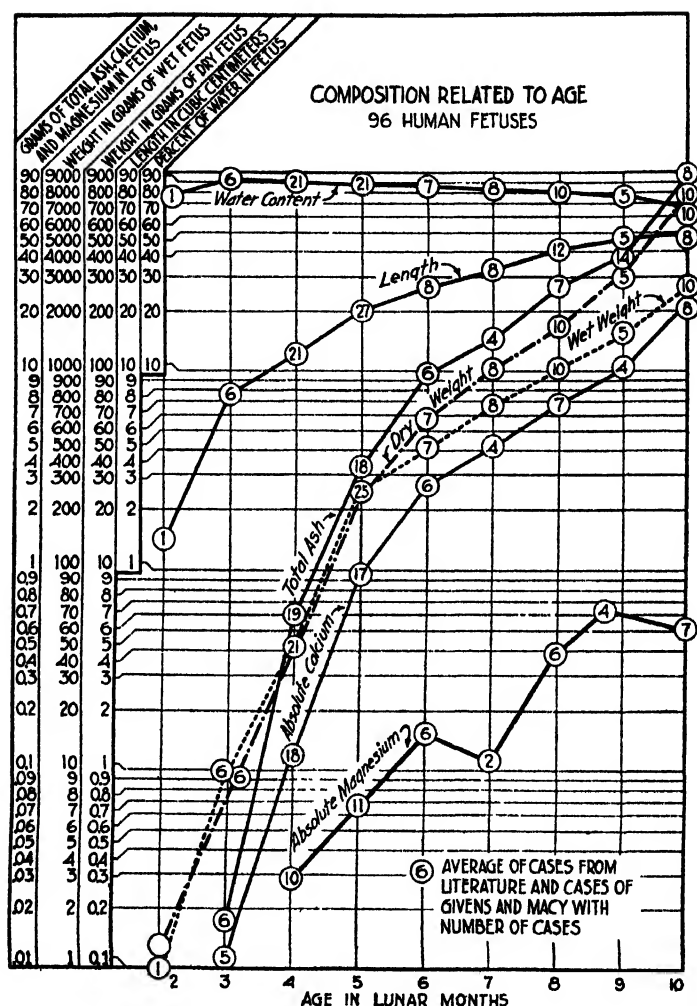
CHART 1. Graph illustrating the relation of age to average chemical composition of the human fetus. The age is plotted in lunar months on the horizontal arithmetic scale and the average length, wet weight, total solids, water content, total ash, calcium, and magnesium on the vertical logarithmic scale. The total number of fetuses from which the averages figures of composition have been obtained for each particular age group is given within circles. This semilogarithmic graph not only shows the absolute value but also the relative rates of change in composition as age *in utero* progresses.

vertical logarithmic scale, thus showing not only the absolute values but the relative rate of change of values throughout intra-uterine life. The number of cases from which the average figures of composition for each particular age group have been obtained is given in circles. In some instances, as in the case of magnesium, in which there are few cases in a particular age group, the irregularities in the curves may be partially explained by the small number of determinations. The length of the embryo increases very rapidly during the first 4 or 5 months, but the rate decreases during the remaining months. The percentage composition of water in the fetus gradually decreases from 92 at the 3rd month to 72 at maturity. From the number of cases presented it is evident that the fetal demand for total solids, total ash, and calcium increases progressively and at approximately the same rate with age during intrauterine life; whereas, magnesium storage increases at a much slower rate.

Growth of the fetus consists primarily in the transformation of inorganic salts, fats, carbohydrates, amino acids, etc., from the maternal blood into new chemical entities which form the fetal tissue. The fetus *in utero* depends on the maternal body not only for its immediate source of supply, but for a means of elimination of waste products of its metabolism. From a study of the calcium and mineral content of the tibiæ secured post mortem in a series of new born infants born to mothers of known nutritional histories, Booher and Hansmann (13) have concluded that with respect to the deposition of the inorganic constituents of its bones the normal human fetus may be regarded as entirely parasitic on the maternal organism, since large differences in calcium and phosphorus intake by the mothers did not seem to affect the degree of calcification of the tibiæ of the new-born. Although the growth of the fetus during the first half of gestation proceeds at a rapid rate, the actual weight of the human fetus is inappreciable when compared with that of the mother, its weight scarcely approximating 50 gm. by the end of the 4th month. It is not, however, until in the last half of pregnancy that a sacrifice on the part of the mother becomes apparent in either activity or body substance. Indeed, this period is characterized by an extraordinarily rapid accretion of tissue for the fetus has increased to a weight varying from 1497 to 3348 gm. at term.

There are wide fluctuations in the total mineral content of individual fetuses within a given age range. Chart 2 illustrates these fluctuations in the total ash content of 69 individual human fetuses at different ages. Furthermore, the average growth
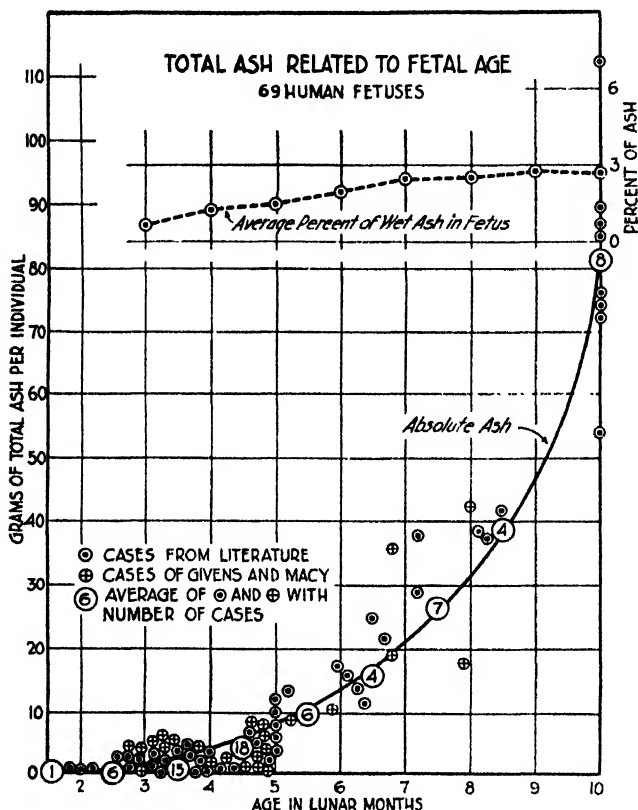

CHART 2. Graph showing the relation of age to total ash, to the per cent of ash in the wet weight, and to the total body solids. The total mineral content of each fetus is given as well as the average composition at different ages. The total number of fetuses from which the average figures of composition have been derived for each age group is given within circles.

curve demonstrates a greatly augmented demand for minerals during the last 2 or 3 months of intrauterine life. The fetus contains on the average only 0.55 gm. of ash by the end of the 4th month, whereas at maturity it may possess from 54 to 112 gm.

The average percentage of ash in the dry fetus rises from 9.7 per cent at the 3rd month to 17.4 per cent at the 6th month, after which it gradually drops to 10 per cent by maturity.   The total
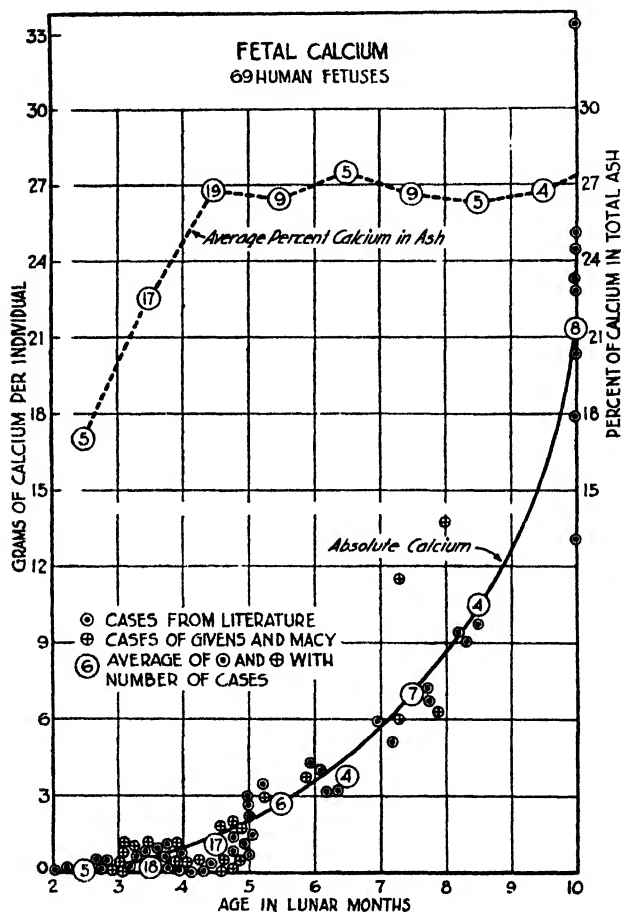

CHART 3.   Graph showing the relation of age to total fetal calcium and to the average per cent of calcium in the ash.   The entire calcium content of each fetus is given as well as the average composition at different ages. The total number of fetuses from which the average figures of calcium content have been derived for each age group is given within circles.

mineral content in the wet fetus increases gradually from 0.73 per cent in the 3rd month to 2.79 per cent at term.

The calcium stored up by the human embryo through the first 4 months of growth averages a little more than 0.1 gm., an inappreciable amount when compared with the 13 to 33 gm. of cal-
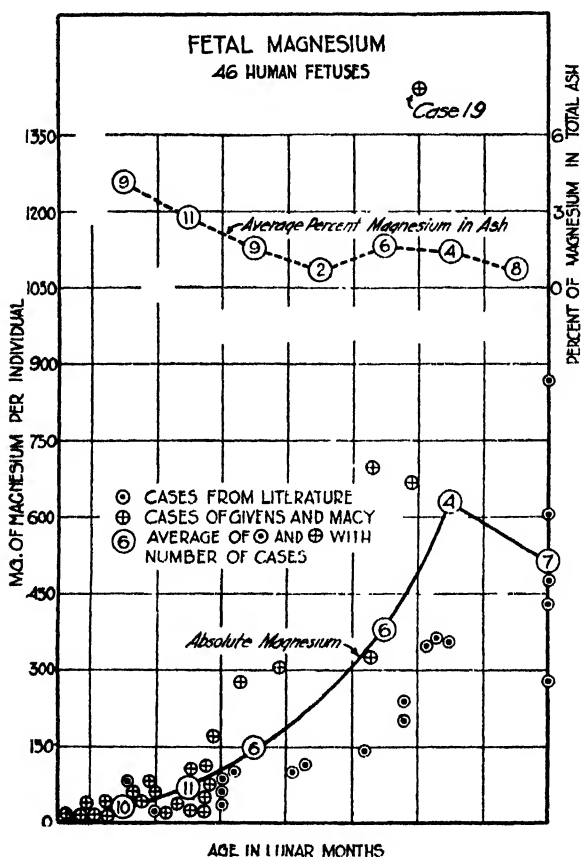

CHART 4.   Graph showing the relation of age to total fetal magnesium and to the average per cent of magnesium in the ash.   The magnesium content of each fetus is given as well as the average composition at different ages.   The total number of fetuses from which the average figures of magnesium content have been derived for each age group is given within circles.

cium fixed in the fetal tissues during the succeeding months.   The actual calcium content of 69 human fetuses is graphically plotted according to age in Chart 3.   Not only is the absolute calcium in

our individual cases and those from the literature given, but the average calcium composition at the different age levels is shown. The number of cases used in obtaining the average curve of growth is given in circles. It is noted that the greatest calcium deposition is made in the last 3 months of fetal life. This is significant when it is recognized that maternal reserves of calcium may become depleted in the last part of gestation as is evidenced by the breaking down or loss of teeth of the mother during or immediately following the termination of pregnancy (14). The percentage of calcium in the total ash of the fetus increases rapidly up through the 4th month and thereafter it remains remarkably constant, varying only between 26 8 and 27.4 per cent. This would indicate that the calcium composition of growth of the fetus is the same during the last months of intrauterine development.

The magnesium per individual and its average composition in forty-six human fetuses are given in Chart 4. The magnesium *in toto* of the fetus at 4 months averages 0.028 gm., whereas at maturity it approximates only 0.5 gm. The average content of magnesium shows an increased utilization up to the 8th month. Since the average composition curve of magnesium is greatly influenced by the large amount of magnesium found in Case 19, it is of importance to note that this premature specimen was one of a pair of twins and lived for 4 days after birth. The food intake and treatment during extrauterine life possibly contributed to its high mineral content. It may be noted that the average percentage of magnesium in the total ash decreases slightly with the age of the embryo.

Robertson (15) has shown that growth in weight or linear dimensions does not proceed with uniformly retarded or accelerated velocity from the moment of its inception until the period of its completion. On the contrary, growth takes place relatively slowly, then more rapidly, and again more slowly, thus yielding an S-shaped curve of growth designated as a growth cycle. Two or three of these sigmoid curves may be superimposed upon one another in the complete growth of an individual. One cycle of man has its inception during the intrauterine growth subsequent to implantation of the embryo and is interrupted by birth when it is not yet half completed and culminates towards the end of the 1st year of postnatal life. The curves illustrating the storage of

minerals in the fetus show the first phase of this sigmoid type curve (Charts 2 to 4). They indicate, furthermore, that in so far as the mother is concerned, the greatest mineral drain on her tissues comes during the last 3 months of gestation, for the fetal demand itself is insignificant up to this time. From the data available, the average monthly increments of the fetus from the 2nd to the 10th months respectively are 0.07, 0.48, 2.64, 6.47, 4.71, 12.12, 12.80, and 41.96 gm. of ash; 0.01, 0.11, 0.83, 1.71, 0.98, 3.16, 3.54, and 12.1 gm. of calcium. It is important that during the last months of pregnancy the mother should demand the best available medical supervision and should follow rigidly the dictum of sound nutritional principles in order that her own body tissues may be preserved and at the same time give to her unborn child materials of sufficient quality and quantity to satisfy optimal growth requirements.

SUMMARY

Twenty-five human fetuses, ranging from 9 to 40 cm. in length and consequently varying from 2 to 8 lunar months in age, have been dried, ashed, and analyzed for calcium and magnesium. These results have been summarized and interpreted together with those in the literature.

The analyses of 96 human fetuses have been used in constructing growth diagrams. By means of a semilogarithmic chart it is demonstrated that the length of the body increases very rapidly during the first 4 or 5 months *in utero*, after which the rate decreases. By the same method it is shown that the fetal demand for total ash, total solids, and calcium increases progressively and at approximately the same rate with age during intrauterine life, whereas magnesium increases at a slower and different rate.

Up to the 4th month the mineral requirement of the fetus approximates *in toto* 0.55 gm. of ash, 0.1 gm. of calcium, and 0.028 gm. of magnesium, whereas by maturity the quantities used up are from 54 to 112 gm. of ash, 13 to 33 gm. of calcium, and 0.277 to 0.78 gm. of magnesium.

The total ash varies from 4 to 21 per cent of the dry body weight. Calcium represents from 6 to 30 per cent of the total mineral content of the body, while magnesium is present in only 0.6 to 0.9 per cent of the total ash.

The greatest fetal demand for minerals comes during the last 3 months of gestation. Whether or not the requirements of the fetus can be met from the mother's food intake is being investigated further.[1]

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THE COLORIMETRIC ESTIMATION OF THE HYDROGEN ION CONCENTRATION OF BLOOD*

By VICTOR C. MYERS, EDWARD MUNTWYLER, DOROTHY BINNS, AND WAYNE H. DANIELSON

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Comparisons of the colorimetric and the hydrogen electrode methods of estimating plasma pH have been reported by a number of workers. Although most workers have conceded that the colorimetric values agree very well with the electrometric pH values when normal plasma is employed, the reliability of the colorimetric method when employed on pathological blood has been questioned. Further, the application of the colorimetric method to dog blood has been seriously criticized (1).

Owing to the ease and rapidity of operation and the small amount of material required, the colorimetric method would appear to be the method of choice for clinical purposes. It has been our hope that the factors causing error with the colorimetric method could be eliminated. The colorimetric estimation may be carried out at room temperature (20°) and corrected to body temperature as described by Cullen (2), or performed directly at body temperature as recommended by Hastings and Sendroy (3). The latter workers found that no correction was necessary when the colorimetric readings were made at body temperature. Stadie, Austin, and Robinson (4) have pointed out that the difference in pH of plasma between 20–38° is subject to change, depending upon the total CO_2, protein concentration, and pH, owing to the fact that with change in temperature the equilibrium of the serum protein-carbonic acid system is shifted. This would obviously

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* A preliminary report of the larger part of this work was presented before the American Society of Biological Chemists at Montreal (Muntwyler, E., and Myers, V. C., *J. Biol. Chem.*, **92**, xlviii (1931)).

introduce a variable correction factor if one were to change the colorimetric reading obtained at 20–38°. An error might arise then if such a procedure were employed with pathological samples showing wide fluctuations in protein concentration, or with experimentally altered solutions (*i.e.*, plasma or serum equilibrated at various CO_2 tensions). Stadie, Austin, and Robinson have discussed the magnitude of this error.

In the present report a comparison was made of the colorimetric values (obtained both at 20° and 38°) and the hydrogen electrode values obtained with unaltered plasma samples equilibrated at definite CO_2 tensions. Further, pH comparisons were made on plasma samples which were altered to give variable protein concentrations. In altering the plasma samples an attempt was made to keep a constant salt concentration while that of the protein was varied, then to equilibrate at definite CO_2 tensions. This was done to determine whether a discrepancy between the colorimetric and hydrogen electrode methods would occur with wide variations in protein concentration and pH.

Methods

The colorimetric pH determinations were carried out as previously described (5). The colorimetric values at 38° were obtained with a bicolorimeter and saline diluent kept in a constant temperature air bath, while the values at 20° were obtained with another bicolorimeter kept in a constant temperature room at 20°. The colorimetric readings were made simultaneously with the determination of the pH by means of the hydrogen electrode at 38°. A modified Clark electrode vessel of 2 cc. capacity was employed. In each instance the same CO_2 tension was introduced with the hydrogen into the electrode vessel as was employed in equilibration. Further, the refill technique was employed as a check.

A given plasma or serum sample was equilibrated at known CO_2 tensions, after which the pH estimations were made. The plasma was analyzed for protein, NaCl, CO_2 at 40 mm. of CO_2 tension, and phosphorus. Dilutions of this plasma or serum were then made with a salt solution, a sodium bicarbonate solution, a pH 7.4 phosphate mixture, and water to give the original concentration of NaCl and phosphate while the protein concentration was

being diluted to either 4 or 2 per cent. The bicarbonate addition was such as to give a 20 mM concentration of total CO_2 under 40 mm. of CO_2 tension. These "diluted" samples were then equilibrated at various CO_2 tensions and the pH determined electrometrically at 38° and colorimetrically at 20° and 38° as though regular plasma samples were being employed (*i.e.*, to obtain the colorimetric value, the samples were diluted 1:21 with

TABLE I

Variations in pH$_c$20° to pH$_c$38° Correction with pH Changes

Material	Sample No	pCO$_2$ (approximate)	pH$_c$38°	pH$_c$20°	pH$_c$20°-pH$_c$38°
		mm.			
True plasma, human	1	20	7 47	7 75	0 28
		75	7 24	7 43	0 19
	2	20	7 38	7 58	0 20
		80	7 14	7 20	0 06
	3	20	7 33	7 53	0 20
		80	7 17	7 26	0 09
	4	20	7 46	7 67	0 21
		80	7 24	7 41	0 17
Serum, human	5	20	7 41	7 76	0 35
		40	7 26	7 51	0 25
		60	7 15	7 39	0 24
	6	20	7 39	7 65	0 26
		40	7 25	7 50	0 25
		60	7 14	7 33	0 21
Plasma, dog	7	20	7 39	7 74	0 35
		40	7 27	7 57	0 30
		60	7 17	7 41	0 24
	8	20	7 61	7 87	0 26
		60	7 27	7 46	0 19

the saline diluent in the cup of the bicolorimeter in exactly the the same manner as with plasma samples).

Results

The results of the pH comparisons are summarized in Tables I to III. Table I shows the variations in the correction pH$_c$20° to pH$_c$38° which were encountered when a given plasma or serum sample was equilibrated at varying CO_2 tensions. With increase in CO_2 tension and consequent lowering of pH the value pH$_c$20°

to pH$_e$38° decreases. It should be pointed out (this will be discussed later) that with increase in CO$_2$ tension the total CO$_2$ is also increased and this affects the above correction in itself.

Table II summarizes the average of a number of values obtained for pH$_c$20° to pH$_e$38°, pH$_c$20° to pH$_e$38°, and pH$_c$38° to

TABLE II

Variations in Various Colorimetric Corrections with Changes in Protein Concentration and pH

pH$_c$38° (range)	pH$_c$20° pH$_c$38°	pH$_c$20°-pH$_c$38°	pH$_c$38° pH$_c$38°	Protein concentration
		Pooled human serum		
7 40-7 50	0 34	0 32	0 01	Undiluted serum
	0 27	0 28	−0 02	Diluted to 4 per cent
	0 16	0 18	−0 02	" " 2 " "
7 30 7 40	0 28	0 25	0 01	Undiluted serum
	0 23	0 25	0 02	Diluted to 4 per cent
7 20-7 30	0 26	0 24	0 01	Undiluted serum
	0 18	0 20	−0 02	Diluted to 4 per cent
	0 12	0 17	−0 04	" " 2 " "
7 10-7 20	0 20	0 20	−0 01	Undiluted serum
	0 15	0 12	0 03	Diluted to 4 per cent
	0 10	0 15	−0 02	" " 2 " "
		Pooled dog plasma		
7 40-7 50	0 26	0 29	−0 04	Undiluted plasma
	0 17	0 26	−0 08	Diluted to 4 per cent
	0 12	0 20	−0 08	" " 2 " "
7 30 7 40	0 29	0 28	0 01	Undiluted plasma
	0 13	0 23	−0 10	Diluted to 4 per cent
	0 09	0 14	−0 04	" " 2 " "
7 20-7 30	0 25	0 24	0 00	Undiluted plasma
	0 12	0 18	−0 05	Diluted to 4 per cent
	0 10	0 12	−0 02	" " 2 " "
7 10-7 20	0 24	0 19	0 05	Undiluted plasma
	0 13	0 17	0 06	Diluted to 4 per cent
	0 08	0 14	0 06	" " 2 " "

pH$_e$38° corrections on CO$_2$-equilibrated human serum and CO$_2$-equilibrated serum which had been altered to give a 2 or 4 per cent protein concentration, while the NaCl and phosphorus concentrations were kept at the original level and the total CO$_2$ close

TABLE III

Comparison of Electrometric and Colorimetric pH Values of Human Serum

Sample No	pH,38°	pH,20° pH,38°	pH,20° pH,38°	pH,38° pH,38°	(CO₂) m-eq	Total protein per cent
1	7 39	0 30	0 23	0 07	24 8	6 8
2	7 42	0 25	0 21	0 04	25 7	5 9
3	7 40	0 26	0 26	0 00	25 0	5 7
4	7 33	0 26	0 26	0 00	19 3	5 8
5	7 38	0 28	0 28	0 00	23 1	5 0
6	7 38	0 27	0 27	0 00	24 4	6 4
7	7 36	0 28	0 25	0 03	22 9	6 6
8	7 44	0 28	0 25	0 03	23 8	5 8
9	7 40	0 27	0 25	0 02	23 1	6 3
10	7 32	0 32	0 31	0 01	20 4	6 6
11	7 39	0 31	0 31	0 00	27 5	6 8
12	7 44	0 32	0 35	-- 0 03	25 1	6 7
13	7 42	0 26	0 23	0 03	28 1	6 2
14	7 43	0 29	0 26	0 03	20 5	5 3
15	7 42	0 25	0 28	− 0 03	22 8	
16	7 39	0 24	0 25	−0 01	21 1	5 5
17	7 41	0 25	0 27	--0 02	23 3	6 3
18	7 41	0 28	0 30	−0 02	26 0	6 6
19	7 41	0 34	0 33	0 01	24 1	7 3
20	7 40	0 28	0 24	0 04	23 0	5 4
21	7 38	0 30	0 23	0 07	25 0	
22	7 41	0 31	0 30	0 01	22 9	
23	7 41	0 31	0 31	0 00	23 3	
24	7 41	0 32	0 27	0 05	23 4	6 8
25	7 42	0 32	0 27	0 05	24 8	6 0
26·	7 35	0 31	0 27	0 04	24 5	6 5
27	7 38	0 32	0 27	0 05	22 0	
28	7 42	0 32	0 27	0 05	26 4	5 2
29	7 41	0 31	0 28	0 03	24 8	5 6
30	7 40	0 33	0 28	0 05	23 5	6 1
31	7 39	0 29	0 25	0 04	27 5	6 1
32	7 41	0 32	0 30	0 02	22 1	6 8
33	7 46	0 29	0 24	0 05	22 8	5 4
34	7 39	0 35	0 31	0 04	26 9	5 4
35	7 39	0 35	0 33	0 02	23 2	6 3
36	7.39	0 32	0 29	0 03	23 3	5 5
37	7 41	0 33	0 34	—0 01	25 2	6 5
38	7 39	0 31	0 25	0 06	30 0	6 4
39	7 39	0 30	0 22	0 08	23 2	5 0
40	7 39	0 32	0 27	0 05	25 4	5 9

TABLE III—*Concluded*

Sample No.	$pH_c38°$	$pH_c20°-pH_c38°$	$pH_c20°-pH_c38°$	$pH_c38°-pH_c38°$	(CO_2)	Total protein
					m.-eq.	*per cent*
41	7 40	0 36	0 27	0 09	26 1	6 6
42	7 45	0 22	0 24	−0 02	22 2	7 2
43	7 42	0 29	0 28	0 01	25.6	7 6
44	7 34	0.30	0 23	0 07		
45	7 36	0 31	0 21	0 10	26 0	5 8
Maximum	7 46	0 36	0 35	0 10	30 0	7 6
Minimum	7 32	0 22	0 21	−0 03	19 3	5 0
Average		0 298	0 27	0 028		

to 20 mm. It will be observed that the value $pH_c20°$ to $pH_c38°$ decreases with a decrease in protein concentration in a given range of pH. Furthermore, as was noted in Table I, the above value decreases with a decrease in pH, resulting from equilibration at a higher CO_2 tension. Similar findings are observed with the value $pH_c20°$ to $pH_c38°$. The correction $pH_c38°$ to $pH_c38°$ remained relatively constant with change in protein concentration and pH.

Table II also presents similar average findings for the $pH_c20°$ to $pH_c38°$, $pH_c20°$ to $pH_c38°$, and $pH_c38°$ to $pH_c38°$ corrections for dog plasma. The $pH_c38°$ to $pH_c38°$ correction with the dog plasma, however, was found less constant.

Recently we have carried out our analyses on serum rather than on oxalated or heparinized plasma. To our surprise the correction $pH_c20°$ to $pH_c38°$ was found to be decidedly higher in this series than had formerly been obtained for plasma, but the explanation for this discrepancy did not become apparent to us until the recent report of Robinson, Price, and Cullen (6) appeared. It will be seen from the data presented in Table III that we have independently obtained essentially the same results as they have now reported.

DISCUSSION

In a previous report on this subject by Myers and Muntwyler (5), in which the colorimetric values at 20° and 38° were compared with the true pH on a considerable number of plasma samples obtained from hospital cases, it was found that 85 per cent were

within ± 0.04 pH of the correct value.   Consequently, it was felt that the colorimetric method gave sufficiently good agreement to make it suitable for clinical use.   It was appreciated at the time, however, that where absolute values were desired the electrometric method should be employed.   It has been our hope that the factors causing the discrepancies between the colorimetric methods and the hydrogen electrode could be elucidated so that the colorimetric method might be used upon various pathological samples without introducing significant error.

Stadie, Austin, and Robinson (4) have pointed out that the difference in pH of plasma between $20\text{-}38°$ is subject to change depending upon the total CO_2, protein concentration, and pH, owing to the fact that with change in temperature the equilibrium of the serum protein-carbonic acid system is shifted.   They were further able to show that with increase in total CO_2 at a given pH and protein concentration the difference in pH between $20\text{--}38°$ decreases.   We have been able to verify this observation on a limited number of determinations on plasma which had been "diluted" with salt solutions to give a 4 per cent protein concentration and a 10 and 30 mM CO_2 concentration.

As may be observed in Table I, when a given sample was altered by increasing the CO_2 tension and consequently lowering its pH, considerable variation in the $pH_e 20°$ to $pH_e 38°$ correction was encountered.   If one accepts the report of Stadie, Austin, and Robinson (4), then in such an alteration one would expect two factors to influence the correction.   Both increasing the total CO_2 and lowering of pH would tend to cause a decrease of the correction $pH_e 20°$ to $pH_e 38°$.   From Table II it will be observed that the $pH_e 20°$ to $pH_e 38°$ and $pH_e 20°$ to $pH_e 38°$ corrections are decreased by decreasing protein concentrations.   It should be pointed out that the total CO_2 was not kept absolutely constant (but close to 20 mM) in these determinations since all CO_2 analyses were balanced against 40 mm. of CO_2 tension.   Consequently the total CO_2 would be less with a tension below 40 mm. of CO_2 and greater with increased CO_2 tensions.   With human serum the $pH_e 38°$ to $pH_e 38°$ correction remained quite constant with changes in protein concentration and pH, although the agreement was not as good in the case of dog plasma with which considerable variation was encountered.   It must be remembered that in all in-

stances the samples were altered in so far as the CO_2 tension was varied or the samples were diluted. Such large variations in the $pH_c20°$ to $pH_c38°$ and $pH_c20°$ to $pH_c38°$ corrections should not be encountered in pH determinations on pathological blood samples. In pathological bloods a lowered pH is usually accompanied by a lowered total CO_2, while an elevated pH generally accompanies an increased total CO_2. These two factors tend to counterbalance each other in affecting the $pH_c20°$ to $pH_c38°$ and $pH_c20°$ to $pH_c38°$ corrections. Hence, it would seem that when one is dealing with plasma in approximately the same pH range, and the same total salt and protein concentration, very reliable results should be obtained since the factors tending to alter the $pH_c20°$ to $pH_c38°$ correction are relatively constant.

The results obtained by diluting plasma or serum as was done in these experiments may not be strictly applicable to what might be obtained with the changing protein concentrations occurring in pathological conditions. In the latter instance the albumin concentrations undergo the major variations, while diluting a sample of given total protein concentration causes an equal change in both albumin and globulin. It is significant, however, that Robinson, Price, and Cullen (6) found in human nephritic serum, in which the albumin was below 4 per cent, that the C' correction was 0.06 to 0.07 pH below that of normal serum.

We know comparatively little about the relative influence of the individual plasma proteins on the colorimetric pH value. In this connection the report of Robinson, Price, and Cullen (6) is of interest. These workers recently observed that the colorimetric correction ($pH_c20°$ to $pH_c38°$) for human *serum* ranges from 0.28 to 0.31. These figures are definitely higher than the values usually accepted for the C' correction in human *plasma*. In Table III we have compared the colorimetric pH determined at both 20° and 38° on forty-five samples collected under oil from hospital patients with the electrometric pH values obtained at 38°. In agreement with the findings of the above workers and also with the earlier observations of Cullen, Keeler, and Robinson (7) the average correction for serum obtained in this series (0.30) was definitely higher than the average C' correction obtained by Myers and Muntwyler (0.22) for human plasma. 80 per cent of the values were within ±0.04 pH of this average value. The correction $pH_c38°$

to pH.38° averaged +0.03 pH, with essentially the same variation as with the pH.20° to pH.38° correction.   Hence, although it would seem that better results should be obtained by performing the colorimetric determination of pH at 38°, practically the same deviation from the true pH was encountered in this series as was observed when the determinations were made at 20°.   It should be pointed out that these samples were not accompanied by marked variations in the acid-base balance.   The maximum variations in pH.38° were from 7.32 to 7.46, for (CO_2) from 19 to 30 milli-equivalents, and for the total serum protein from 5 to 7.6 per cent.

Kydd and Peters (8) have recently criticized the conclusion of Muntwyler, Limbach, Bill, and Myers (9) with regard to the slight elevation of the pH in pregnancy, on the basis that their findings in pregnancy fell within essentially the same range as those reported by Earle and Cullen (10) for normals.   Earle and Cullen carried out their analyses on serum and used a C correction of 0.23, which Robinson, Price, and Cullen (6) now find, as mentioned above, should be 0.30.   This would lower the values reported by Earle and Cullen by 0.07 pH and would thus completely invalidate the criticism of Kydd and Peters (8) on this point.

SUMMARY

A comparison has been made of the colorimetric pH determined at 20° and 38° with the true pH determined electrometrically at 38° on plasma and serum samples which had been experimentally altered to ascertain whether with such alteration a variation in the colorimetric corrections would be found.

Equilibrating a plasma or serum sample with different CO_2 tensions was found to cause a variation in the pH.20° to pH.38° correction.   With increasing CO_2 tensions (lowering of pH and increase in total CO_2) this correction was found to decrease.   By diluting plasma or serum with salt solutions so that the total protein concentration was varied but the salt concentration maintained at a relatively constant level, the pH.20° to pH.38° correction was observed to decrease with a decrease in the protein concentration in a given pH range.

A comparison of forty-five serum samples obtained from hospital patients gave a higher colorimetric correction (pH.20° to pH.38°)

than had previously been obtained for human plasma; namely, an average of 0.30 pH.   This is in agreement with the recent work of Robinson, Price, and Cullen.   The correction $pH_c38°$ to $pH_s38°$, of Hastings, obtained in this same series of samples averaged $+0.03$ pH.   The variation was essentially the same as was obtained when the new colorimetric correction factor (0.30) for serum was employed.

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THE QUANTITATIVE MICROANALYSIS OF PLANT JUICE FOR REDUCING SUGARS AND SUCROSE*

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The use of expressed plant juice in metabolic studies has made it necessary to employ microchemical methods for the estimation of the constituents found in the fluid of growing plant tissue. In any research in which the quantity of available juice is limited it follows that the quantities of juice components will be correspondingly small. For the estimation of these substances resort must be made to microvolumetric or microcolorimetric methods. In general both of these procedures demand a clear colorless solution. The preparation of a clarified juice sample requires the use of reagents which usually produce, with the color pigments and proteins present, large amounts of precipitate. This introduces the necessity of choosing precipitating reagents from two points of view. These reagents must not precipitate the substances to be determined nor must the precipitate formed remove such substances by adsorption. It is the purpose of this paper to demonstrate a means for the clarification of plant juice and the determination of microamounts of reducing sugars and sucrose in small aliquots of plant juice.

Methods

For the determination of sugar the volumetric method of Hagedorn and Jensen (4), the micromethod of Folin (1), a modification of this method, and the copper method used by the same author (2) were compared. In the microcolorimetric method the directions call for the use of sodium cyanide as an aid in the reduction, which is obviously an undesirable reagent to work with. It was

found that if the carbonate and potassium ferricyanide were united (similar to the Hagedorn and Jensen solution) sufficient reduction took place so that the color developed could be compared in a colorimeter. This final solution contained 3 gm. of recrystallized potassium ferricyanide and 10 gm. of anhydrous sodium carbonate per 500 ml.; 2 ml. were used for each determination.[1]

Folin sugar tubes were found to be convenient for the colorimetric methods and large (8 × 1 inches) test-tubes for the volumetric method.

Folin and Malmros (3) advise the use of a yellow light filter with the micromethod. Because of the type of color which results from the combination (filter plus blue solution) the light filter was also used with the copper method.

Juice was obtained by enclosing samples tightly in cheese-cloth, freezing with a stream of carbon dioxide, allowing the excess carbon dioxide to escape from the sample, and then expressing the juice by means of a hydraulic press.

EXPERIMENTAL

For the clarification of juice samples it was found that a slight excess of a saturated neutral lead acetate solution was sufficient to precipitate the coloring matter and proteins. The excess lead can be taken from solution by adding disodium phosphate. To show the effect of these reagents on a sugar solution, determinations were made by all the sugar methods mentioned above. The recoveries were between 96.25 and 101.00 per cent for both glucose and sucrose, indicating no appreciable loss due to clarification.

As fructose is present after inversion of sucrose, determinations were run and its reduction value proved to be 98.0 per cent, which is close enough to the theoretical value to make the difference negligible.

A study of the adherence of the colorimetric methods to Beer's law is shown in Fig. 1. The three procedures show a rather good agreement over a fairly large range; however, if a single standard is to be used corrections are necessary.

[1] The modified microprocedure is exactly the same as the original Folin micromethod, except that the new alkaline ferricyanide solution is substituted for the sodium cyanide and the potassium ferricyanide, sodium carbonate solutions.

The reduction values with the Hagedorn and Jensen method for the range including 0.1 to 0.5 mg. can be calculated from the following equation: $y = 0.1562 + 5.522\,x$, in which $y = $ ml. of 0.005 N sodium thiosulfate and $x = $ mg. of glucose.

For the determination of sucrose the solution is made acid to methyl red, 2 drops of a 0.5 per cent invertase solution added, and the mixture kept at room temperature or slightly above for 2 hours. By using pure sucrose (Bureau of Standards sucrose $[\alpha]_{546\ 1\ m\mu}^{20\ 0} = 78°.\ 342)$ these conditions proved to be adequate for
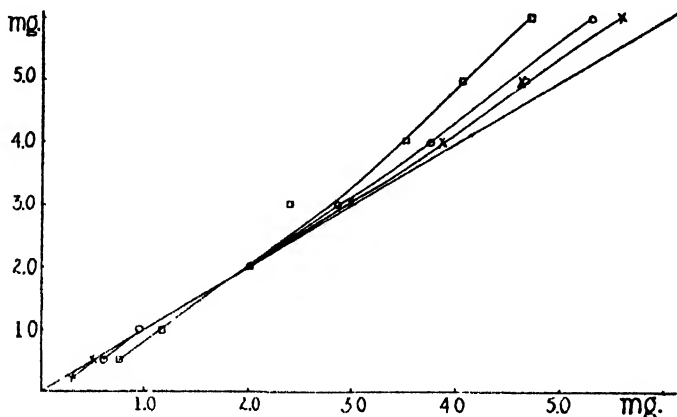

Fig. 1. A graphical representation showing agreement of the methods employed with Beer's law. □ = Folin's micromethod; × = Folin's modified micromethod; ○ Folin's copper method. The abscissa scale denotes the glucose recovered; the ordinate scale, the glucose present.

complete inversion. Fifteen determinations gave an average inversion of 99.8 per cent.

General Procedure Adopted—A sample of juice, usually 1 or 2 ml., is pipetted into a centrifuge tube (15 or 50 ml.) and diluted to 10 ml. Saturated neutral lead acetate is added, 1 or 2 ml. being sufficient, and the tube is centrifuged. Add 10 per cent disodium phosphate solution until the sugar solution is blue to brom-thymol blue, dilute to volume, and again centrifuge.

An aliquot, depending on the amount of sucrose present, is transferred to a Folin sugar tube, made acid to methyl red, and 2 drops of a 0.5 per cent Wallerstein's invertase scale preparation

added. To determine the amount of dilute acid needed to bring the sample to the proper pH value, use a trial sample, for methyl red in the presence of the resulting Prussian blue imparts an off shade to the solution. After standing for 2 hours at room temperature, the solution is made alkaline (pH 8 is sufficient) and the determination is made. Standards containing 2 drops of invertase and equivalent amounts of either glucose or inverted sucrose can be used. The Hagedorn and Jensen method cannot be used, since the presence of invertase has an effect on the determination.

TABLE I

Sugars in Plant Juice

The results are expressed in mg. per ml. of juice.

	Reducing sugars				Sucrose		
	Folin cyanide	Modified Folin cyanide	Folin copper	Hagedorn and Jensen	Folin cyanide	Modified Folin cyanide	Folin copper
Apple	18 90	18 90	17 28	17 52			
	12 60	12 60	8 00	11 57			
Beet	21 24	21 43	21 43	21 90	0 98	0 97	1 15
	10 00	10 10	10 17	8 58	11 60	10 88	11 23
Celery	1 44	2 36	1 03	2 56	17 79	18 66	12 91
	2 94	3 65	1 87	3 93	15 80	18 55	17 04
Chrysanthemum	3 00	3 75	2 40	2 52	15 40	15 64	14 07
Lettuce	8 26	8 22	8 22	8 03	10 43	10 25	9 07
	8 46	10 07	7 87	7 66	19 52	27 90	18 65
Tomato	7 60	9 40	8 26	8 61	17 71	20 90	13 50
	1 28	1 97	0 54	1 44		18 55	15 28
	1 69	2 46	1 02	2 06	14 71	22 03	12 50

For the estimation of reducing sugars an aliquot of 1 or 2 ml. is transferred to a Folin sugar tube or test-tube and determined according to the method most desirable. A series of standards is prepared, or a correction chart is used to give more accurate figures.

Results

The procedure just described and the aforementioned methods were applied to various plant juices. In all the different plant extracts used, it was necessary to add sucrose, as growing conditions were such that the plants did not manufacture this carbo-

hydrate. The sucrose was always added before the juice was cleared. The results with various plants are listed in Table I.

The sugar content as determined by Folin's copper method appears to be the one more nearly correct, if the reasoning is based on the general assumption that the lower sugar values are the correct ones. The reason for the low values is due probably to the fact that the alkaline copper tartrate reagent is less sensitive than the ferricyanide solution. This is true, for small amounts of sugar (0.01 to 0.2 mg.) can be determined by the ferricyanide, while the most favorable range for the copper solution is from 0.2 to 0.4 mg. of glucose. As there is possibly present in plant juice a certain amount of non-sugar reducing substances, Folin's copper solution would not be affected by them, whereas they would reduce the more sensitive ferricyanide.

In a recent paper Munday and Seibert (5) have shown that the Hagedorn and Jensen determination is sensitive to substances of low reducing power, such as certain amino acids. They also showed that their precipitating reagents failed to remove these interfering substances completely from solution. It seemed obvious then that this might account for the high values for the Hagedorn and Jensen method as presented in Table I. This was not the case however, for the biuret. Millon, xanthoproteic, and Adamkiewicz tests on completely clarified juice were all negative, indicating that some other non-glucose reducing substance was responsible.

SUMMARY

1. A method for the clarification of plant juice has been developed which is effective even when highly colored juices, such as those obtained from beet tissue, are used.

2. Three colorimetric methods and one volumetric method have been applied to the clarified juice sample.

3. Of these methods, Folin's with the alkaline copper tartrate reagent appears to give the best results.

4. The difference in sugar values is not due to those amino acids which reduce sugar reagents, for tests show the absence of amino acids in the lead-free filtrates.

5. The discrepancy must then be due to the presence of some other type of non-glucose reducing substance.

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STUDIES IN ACETYLATION. THE FATE OF p-AMINOBENZOIC ACID IN THE RABBIT

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Acetylation as a means of detoxication is not very common. So far it has been observed in the case of amino compounds only (1). p-Aminobenzoic acid, for example, is partially acetylated by the rabbit and eliminated as p-acetylaminobenzoic acid (2). It is conceivable that the acetyl group may be derived from carbohydrates, fats, or even proteins. In order to study this mechanism somewhat more in detail, the effect of the injection of insulin upon the acetylation process was examined. The observation of du Vigneaud (3) that insulin is inactivated by glutathione led us to extend our observations to the effect of the simultaneous injections of these substances upon the acetylation process.[1]

EXPERIMENTAL

Female rabbits weighing 1 to 2 kilos were maintained on diets of 75 gm. of oats and 100 gm. of cabbage daily. They were injected subcutaneously with the p-aminobenzoic acid (in the form of the sodium salt), the 48 hour sample of urine collected, and the acetylated compound isolated. Insulin, together with the p-aminobenzoic acid, was next injected, then the glutathione plus the acid, and finally the insulin, glutathione, and the acid. For the sake of economy of space, the results are compressed into one table (Table I). The intervals between injections were approxi-

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[1] We have for some time past been of the opinion that one of the functions of glutathione may be its detoxicating property, inasmuch as all three of its amino acids are typical detoxicating agents of the body.

mately 1 week. During the course of the experiment, the weights
of the rabbits varied from 1.3 to 2.2 kilos. The total nitrogen in
urine (in gm. per 48 hours) varied as follows: Rabbit 1, 1.10 to
2.00; Rabbit 2, 1.00 to 1.25; Rabbit 3, 1.46 to 2.21; Rabbit 4, 1.03
to 1.91; Rabbit 5, 1.52 to 2.00. To make clear certain points in
Table I, we call attention to the second column. When 1 gm. of
the *p*-aminobenzoic acid was injected, 0.30 gm. of the acetylated
compound was excreted. This was followed by a 2 gm. injection

TABLE I

Effect of Injection of p-Aminobenzoic Acid, Insulin, and Reduced Glutathione

p-Aminobenzoic acid injected	Excreted as p-acetylaminobenzoic acid (gm per 48 hrs)				
	Rabbit 1	Rabbit 2	Rabbit 3	Rabbit 4	Rabbit 5
gm.					
1 0	0 30, 0 28	0 15, 0 16	0 32, 0 29	0 10	0 17
2 0	0 65, 0 50	0 20, 0 21	0 40, 0 42	0 15	0 24
1 0 + insulin*	0 62	0 28	0 57	0 16	0 29
2 0 + "	1 02	Died	1 18	Died	0 40
1 0 + reduced gluta-thione†	0 29		0 30		0 16
2 0 + reduced gluta-thione†	0 55		0 41		0 23
1 0 + insulin and glu-tathione	0 44		0 30		0 19
2 0 + insulin and glu-tathione	0 55		0 57		0 27

    * 0 5 unit per kilo of body weight.
    † 5 0 mg. per kilo of body weight.

of *p*-aminobenzoic acid and an excretion of 0.65 gm. of the acety-
lated compound. Again 1 gm. of *p*-aminobenzoic acid was in-
jected and 0.28 gm. of the acetylated compound was eliminated.
This was followed by an injection of 2 gm. of *p*-aminobenzoic acid
and an elimination of 0.50 gm. of the acetylated compound. The
first two lines in the third and fourth columns are to be viewed
similarly.

For the isolation of the acetylated compound, the 48 hour
sample of urine, preserved with toluene, was neutralized, evapo-
rated on a steam bath to a thick syrup, cooled, acidified to Congo

red with sulfuric acid, and finally extracted with ether for 10 hours in a continuous extractor.[2]   The ether extract was evaporated to dryness on a steam bath and the residue taken up with 10 cc. of water.   Enough 3 N hydrochloric acid was now added to convert any unchanged p-aminobenzoic acid into its water-soluble hydrochloride.   The insoluble acetylated compound was filtered and recrystallized from boiling water and norit.   The product was filtered, dried at 60°, and its melting point determined.   A mixed melting point (with some of the acetylated compound obtained from the Eastman Kodak Company) was also determined.   The melting point was found to be 250°.   The nitrogen determined was 7.78 per cent and 7.80 per cent; calculated 7.82 per cent.

<div align="center">DISCUSSION</div>

It is quite obvious that insulin markedly increases the output of the acetylated compound.   That this is the result of a stimulating effect upon carbohydrate metabolism seems probable.   It further emphasizes the probable importance of acetaldehyde as an intermediate product in carbohydrate (and fat?) metabolism.   Reduced glutathione[3] alone, on the other hand, has no effect upon the acetylation process.   But, in striking confirmation of du Vigneaud's *in vitro* experiments, the simultaneous injections of insulin and glutathione very definitely inhibit the output of p-acetylaminobenzoic acid.   Such a result, as du Vigneaud indicates, is probably due to the inactivation of insulin by glutathione.

Approximately 75 per cent of the original p-aminobenzoic acid has still to be accounted for.   On an average, for 1 gm. of the acid injected an amount of acetylated product is recovered in the urine which, calculated as p-aminobenzoic acid, amounts to about 0.25 gm.   Undoubtedly some of the acid is eliminated in the form of the glucuronate.   Indeed, after removing the acetylated product with ether, the residue gives a very striking color test with naph-

thoresorcinol. This is in accord with the views of Quick (4), who, however, overlooks the possibility of acetylation.

SUMMARY

1. *p*-Aminobenzoic acid is acetylated by the rabbit to the extent of about 25 per cent.

2. The injection of insulin (0.5 unit per kilo of body weight) markedly increases the output of the acetylated compound.

3. Reduced glutathione (5 mg. per kilo of body weight) does not affect the acetylation process.

4. The simultaneous (but separate) injections of insulin and reduced glutathione produce a decrease in the amount of acetylated product as compared to the injection of insulin alone, thus indicating an inhibition of insulin activity by reduced glutathione *in vivo*.

5. The probable bearing of these results on intermediate metabolism is stressed.

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THE CONCENTRATION AND PROBABLE CHEMICAL NATURE OF VITAMIN G

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Attempts to concentrate and isolate or identify vitamin G have disclosed many experimental difficulties which have greatly retarded progress in the investigations of the chemical nature of vitamin G. It would seem that these difficulties are now sufficiently well recognized as not to require further elaboration here.

Most investigators active in the study of the chemical nature of vitamin G (B_2) are of the opinion that vitamin G is a nitrogenous compound and certain of these believe that it may also contain sulfur.

Very recently Kuhn and his coworkers (1, 2) have reported the isolation of the yellow pigment from egg white which they have named *Ovoflavin* and which they believe to be one factor of the vitamin G (B_2) complex. This pigment was obtained by a method including adsorption and precipitation procedures, in crystalline form; elementary analysis indicated an empirical formula, $C_6H_{10}N_2O_3$. In their second publication they report that this pigment substance requires a further supplementary fraction obtained from autoclaved yeast in order to promote growth in vitamin G test rats.

Previous work (3) in this laboratory has shown that a 10-fold concentrate of the vitamin G in skim milk powder may be prepared by a simple extraction with boiling 93 to 94 per cent (by weight) ethyl alcohol. An attempt to concentrate the vitamin further by introduction of an ethyl ether precipitation procedure furnished evidence indicating that a separation of vitamin G factors had occurred at this step.

The present study was designed to introduce only the mildest chemical operations, avoiding particularly precipitation proce-

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dures, in hopes that a much greater concentration of vitamin G might be effected before a separation of factors was encountered.

A very simple means is here described by which a concentrate carrying 900 to 1000 vitamin G units per gm. (corresponding to a potency of the order of 2000-fold that of fresh whole milk) was obtained from whey powder. Yields of 1.5 gm. were obtained from 500 gm. of the whey powder and in so far as present knowledge enables one to judge, there was no separation of vitamin G components.

In all cases the vitamin G values were measured under conditions which practically eliminated the influence of coprophagy, and under these conditions the reliability and precision of the measurements were as satisfactory as the rat growth method for other vitamins.

<center>EXPERIMENTAL</center>

A commercial whey powder from which a considerable portion of the lactose had been removed was obtained through the courtesy of Dr. L. A. Rogers (Dairy Bureau, United States Department of Agriculture) and was used as the starting material for the concentration of vitamin G. Partial analysis of this whey powder indicated the following proportions of constituents: moisture 2.7 per cent, ash 14.35 per cent, phosphorus 1.07 per cent, nitrogen 5.7 per cent, and lactose 52.7 per cent. This whey powder carried 20 Bourquin-Sherman (4) vitamin G units per gm.

A boiling ethyl alcohol extraction of the whey powder was conducted essentially after the manner employed earlier by Page (3) in the extraction of skim milk powder, except that the combined extracts were chilled and the lactose which separated out was discarded. The extract was then reduced to about 1 liter by vacuum distillation (40–50°) and thoroughly dried in an evacuated desiccator over sulfuric acid. The alcohol extract was yellow, green-fluorescent; the desiccated solids were shiny bright yellow, carried considerable lipid material, and were granular, due to the relatively large proportions of lactose. Three different preparations each made from 500 gm. portions of the whey powder yielded 43, 42, and 38 gm. respectively of alcohol-extracted solids; i.e., about 8 per cent of the solids of the whey powder were extracted. The three products were tested for vitamin G potency and were

found to contain 108, 80, and 123 vitamin G units (Bourquin and Sherman (4)) per gm. respectively. Partial analysis of the first preparation indicated the following proportions of constituents: moisture (40°, vacuum oven) 0.7, ash 15.94, phosphorus 0.24, nitrogen 2.8, and lactose 51.0 per cent. The Liebermann-Burchard test for cholesterol was positive and the biuret test weakly positive. The nitrogen content of the second and third preparations was 2.6 and 3.0 per cent, respectively; i.e., the vitamin G activity and the nitrogen content of the three preparations varied in the same order. The alcohol-extracted residue resulting from the first preparation (corresponding to the extracted solids containing 2.8 per cent nitrogen) was found to contain 12 vitamin G units per gm. From these results calculation of the vitamin G recoveries shows that none of the vitamin G activity of the whey powder had been destroyed in this extraction, for approximately 4600 units were carried by the extract and approximately 5500 units remained in the extracted residue.

Further experimentation showed that a considerable part of the vitamin G activity in the dried alcohol-extracted solids could be removed by extraction with a mixture of chloroform and ethyl alcohol (2:1 by volume), thus largely eliminating the lactose which threatened to be a troublesome factor in further manipulations directed toward increasing the concentration of vitamin G. This procedure, however, served also to concentrate the lipids to such an extent that the vitamin G potency was not much improved over that of the alcohol-extracted solids. That the riddance of most of the lactose was an important step forward will be apparent later.

When 42 gm. of alcohol-extracted solids from the whey powder were extracted with 300, 300, and 120 cc. respectively of a chloroform-ethyl alcohol (2:1 by volume) mixture by refluxing in the presence of a small stream of purified nitrogen to agitate the system and reduce the possibility of oxidation processes, a bright yellow, green-fluorescent extract was obtained. The extracts were filtered on a small Buchner funnel; the combined extracts were then reduced in volume by use of a suction pump attached to a vacuum type desiccator or by drawing a stream of purified nitrogen over them until the odor of chloroform was absent, the last traces of alcohol being removed in an evacuated desiccator con-

taining sulfuric acid. By this means about 25 per cent of the solids of the alcohol extract were removed; *i.e.*, 9 to 10 gm. of the solids extracted by the boiling 93 to 94 per cent alcohol were obtained in the chloroform-alcohol extract. A preparation carried to this step indicated a vitamin G potency of 125 units per gm. while the corresponding extracted residue left behind carried 42 units per gm. The chloroform-alcohol extract and its residue, therefore, could account for only about 2400 units (9 gm. × 125 units + 31 gm. × 42 units) of the 4000 to 4500 vitamin G units carried by the material extracted Attempts to explain this loss of vitamin activity on the basis of factor separation were not successful, since no supplementary relationship could be demonstrated between the chloroform-alcohol extract and the corresponding extracted residue. The dried solids extracted with the chloroform-alcohol mixture were obviously inhomogeneous in nature; they contained a large porportion of lipids and clumps of orange-red pigment material. The nitrogen content of this product was 3.0 per cent. The Liebermann-Burchard test was strongly positive. It will be noted that the vitamin G potency of the boiling alcohol-extracted preparation containing 3.0 per cent nitrogen was essentially the same as the vitamin G potency of this product which contained the same percentage of nitrogen.

The solids extracted with the chloroform-alcohol mixture were next subjected to extraction with purified anhydrous ethyl ether in order to remove the inert lipids. Two separate products of 9 to 10 gm. each of chloroform-alcohol-extracted solids (corresponding to about 40 gm. of boiling alcohol-extracted solids or 500 gm. of whey powder) were extracted with anhydrous ether in a Soxhlet for 17 hours, and in each case yields of about 1.5 gm. were obtained. These ether-extracted residues were orange-red in color and very hydroscopic; dilute water solutions of the products were readily decolorized by visible light. In so far as practical all the extraction processes and preparations were shielded from excessive light. The nitrogen content (Pregl-micro-Kjeldahl) of the two preparations was 6.5 and 8.5 per cent, respectively, and the vitamin G values 885 and 1000 units per gm., respectively. It is apparent from these results that extraction with purified ether conserved all the vitamin G activity of the chloroform-alcohol-extracted material, and that 13 to 16 per cent of the vitamin G

activity of 500 gm. of the original whey powder was concentrated in 1.5 gm. of ether-extracted residue. In other words, by three simple extraction processes a vitamin G concentrate containing 900 to 1000 Bourquin-Sherman units per gm. has been prepared from commercial whey powder corresponding to a 40- to 50-fold concentration of the vitamin G in the whey powder (see Table I). This concentrate represents a vitamin G potency of the order of

TABLE I

Summary of Vitamin G Potencies, Vitamin G Recoveries, and Yields of Concentrates Prepared from Whey Powder

Preparation	Yield	Vitamin G measurement 4 wk. test period			Vitamin G potency	Total vitamin G in preparation
		No. of rats	Supplement per day	Average weekly gain*		
	gm.		*mg.*	*gm.*	*units per gm.*	*units*
Original whey powder	500†	6	260	11 2 ± 0.6		
		4	85	5 0 ± 0 3	20	10,000
Solids of boiling alcohol extract	43	6	39	8 6 ± 0 8		
		4	13	4 2 ± 1 5	108	4,640
	42	4	13	2 9 ± 0 7	80	3,360
	38	6	13	4 8 ± 2 2	123	4,670
Boiling alcohol-extracted residue	457	4	170	6 3 ± 0 6	12	5,480
Solids of chloroform-alcohol extract	8 8	4	13	4 8 ± 1 1	125	1,100
Chloroform-alcohol-extracted residue	31	4	26	3 3 ± 0 2	42	1,300
Ether-extracted residue	1 46	6	1 7	4.6 ± 1 0	885	1,300
	1 59	4	1.2	3 6 ± 0 4	1000	1,600

* The figures following the plus-minus signs show the average deviation.

† The initial starting material for each preparation was 500 gm. of the whey powder.

2000-fold greater than that of fresh whole milk. According to unpublished data (Booher and Blodgett) fresh whole milk contains about 0.5 vitamin G unit per gm.

3 to 4 mg. of the ether-extracted residue were fed as a vitamin G supplement to eight rats which had previously been deprived of vitamin G for 8 weeks subsequent to weaning. This supplement induced an average growth rate of 7.0 ± 2.4 gm. per week over a

4 weeks period.   It had been suggested previously (3) that, in
the absence of a second growth-limiting vitamin G component
(Bourquin-Sherman diet) in the supplement, resumption of growth
might not take place under these conditions.   Since there was no
slackening in the growth rate with the usual method of measuring
the vitamin G value of the concentrate and in view of the experi-
ment just described, it is concluded that the ether-extracted residue
is a concentrate of vitamin G, as a whole, in so far as its growth-
promoting qualities are concerned.

The ether-extracted residue, containing 1000 vitamin G units
per gm., when fed as a vitamin B (B_1) supplement to four vitamin
B_1-depleted rats (Chase-Sherman (5) diet) at levels of 3.2 mg. per
rat per day did not show any appreciable improvement over results
with control animals receiving no supplement.

A summary of the vitamin G potencies, vitamin G recoveries,
and yields of the concentrates and residues from whey powder
mentioned in this report is shown in Table I.

The final vitamin G concentrate which, in daily doses of 1.2 mg.,
induced a growth response of 3.6 ± 0.4 gm. per week in vitamin G
test animals and which apparently did not carry significant
amounts of vitamin B (B_1) nor indicate that there was a separation
of vitamin G components may be characterized by the following:
(1) as a solid the concentrate was orange-red in color and very
hydroscopic; (2) solutions of the concentrate in water, 93 to 94
per cent hot alcohol, and chloroform-alcohol mixtures (2:1 by
volume) were yellow, green-fluorescent; (3) dilute water solutions
of the concentrate exposed in a test-tube to sunlight were rapidly
decolorized; (4) the intensity of the yellow color of the concentrate
is greater in 0.1 N sodium hydroxide than in water, the color almost
wholly disappears (reversibly) in 0.1 N hydrochloric acid, but is not
appreciably affected by 0.1 N acetic acid; (5) the concentrate con-
tained 8.5 per cent nitrogen; (6) the test for sulfur was positive,
although the possibility of contamination cannot be excluded with
certainty; (7) silver nitrate added to a dilute water solution of the
concentrate carried down the yellow pigment, producing a floccu-
lent precipitate with a slightly reddish tinge; (8) a slight precipitate
appears on addition of a few drops of 10 per cent lead acetate
(neutral) to a 0.10 per cent solution of the concentrate in water;
(9) the biuret test is negative; and (10) spectrographic examination

of the pigmented concentrate (dilute water solutions) showed only a general absorption throughout the region of 2000 to 5000 Å., the absorption decreasing with increase of the wave-length. Many of the general properties of this concentrate are similar to those reported for *Ovoflavin*. The general properties of the vitamin G concentrate are in excellent agreement with those of lactochrome of whey as first described by Blyth (6) in 1879 and later studied by Bleyer and Kallmann (7) and others (8, 9).

SUMMARY

Experimental evidence is presented pointing to the probable identity of vitamin G and the water-soluble yellow, green-fluorescent pigment of whey. The chemical properties of a vitamin G concentrate obtained from whey powder and carrying 1000 vitamin G "units" per gm., corresponding to a 40- to 50-fold concentration of vitamin G in whey powder from which a large fraction of the lactose had been removed or to a 2000-fold concentration of vitamin G in fresh whole milk, are in good agreement with the properties of lactochrome in so far as knowledge of these is available. Although this vitamin G concentrate is not pure lactochrome, it is evident that lactochrome is its major constituent.

Measurement of the vitamin G value of this concentrate showed it to be active in somewhat less than 1 mg. doses but indicated no separation of vitamin G components. The concentrate did not carry any significant amounts of vitamin B (B_1).

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A NOTE ON THE DETERMINATION OF LACTIC AND PYRUVIC ACIDS

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In order to determine satisfactorily the small quantities of lactic acid present in the amounts of blood obtainable from small animals, it has been found necessary to modify somewhat the procedure of Friedemann, Cotonio, and Shaffer (1), so as to reduce a variable blank originating from rubber connections of the apparatus, from reagents, and from laboratory air, and also to increase the sensitiveness of the titration end-point by decreasing the volume of solution in which the bound bisulfite is titrated.

With a resting blood lactate level of 10 mg. per cent, the filtrate representing 0.5 cc. of blood (0.05 mg. of lactic acid) yields a titration of only 0.22 cc. of $0.005 \text{ N } I_2$. Without special precautions the blank on reagents with the usual apparatus and room air often amounts to half this quantity. With a modified apparatus and purified reagents the blank may be reduced to about 0.02 cc., thus permitting analysis with amounts of blood filtrate representing 0.5 to 1.0 cc. of blood.

Apparatus[1]—An apparatus which meets these requirements is shown in Fig. 1. It consists of a West indented reflux condenser (2) attached to an all-glass distillation flask and head similar to that used by Mawson and Ritchie (3), and a more efficient absorption tower, which, if properly constructed and thoroughly cleaned, drains perfectly and requires only 3 to 4 cc. of wash water (see also

Davenport and Davenport (4)).   The indentations in the absorption tower, which are in two planes and are opposite each other in each plane, should be separated on the inside by 2 to 3 mm.   If they are closer, draining will not be satisfactory.   Short pieces of
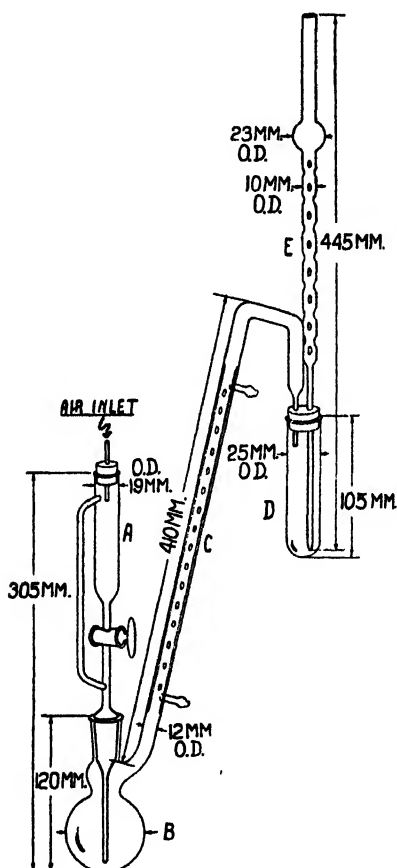
Fig. 1. Apparatus for determining the lactic acid present in small amounts of blood.   A is the reservoir; B, the boiling flask; C, the condenser; D, the receiving tube; and E, the absorption tower.   All measurements are outside dimensions.

rubber tubing are used for making connections with glass tubing to lead washed air, CO_2, or nitrogen into the head.   For this purpose *pure gum tubing* should be used.   We use tank CO_2, with

which washing is unnecessary. If room air containing SO_2 or bisulfite-binding substances is used, it should be washed with bisulfite followed by alkali (to retain SO_2 from the bisulfite). The presence of SO_2 in the boiling flask may lead to low results due to retention of acetaldehyde.

Determination—20 to 30 cc. of lactic acid solution, together with a pinch of talc and 5 cc. of the acid-manganous sulfate reagent (0.5 N H_2SO_4, M Na_2SO_4, and 5 per cent $MnSO_4$), are introduced into the boiling flask, *B*. 5 cc. of 0.01 N $KMnO_4$ are placed in the reservoir, *A*. 2 cc. of 1 per cent $NaHSO_3$ (2 per cent if 1.0 mg. or more of lactic acid is expected) are measured into the receiving tube, *D*. Moderate suction is applied to the upper end of the absorption tower, *E*. Water is run through the condenser, *C*, at such a rate as to maintain a temperature of 25–30° at the outlet during the aeration. A microburner is used to heat the boiling flask. After the solution has come to a boil, the stop-cock is opened just enough to permit the permanganate solution to drip slowly through the tube below. After boiling and aerating 25 to 30 minutes, the burner is removed and the suction stopped. The absorption tower is washed twice (into the receiving tube) with 2 cc. portions of water. The greater part of the excess bisulfite is oxidized with 0.3 to 0.4 N iodine in order to keep the volume of solution as small as possible. Final adjustment of the first end-point and estimation of the bound bisulfite are carried out in the usual way with 0.01 or 0.005 N iodine solution. To remove excess iodine, when the first end-point is overrun, a few drops of dilute bisulfite solution are used. A microburette carrying a detachable capillary glass tip is used for the titrations.

The residue in the boiling flask is removed by suction.

Recovery of Lactic Acid—A sample of zinc lactate, which by the macroprocedure gave 95.4 per cent of the theoretical iodine consumption, was analyzed by the microprocedure with the following results: 2.25 mg. of lactic acid gave 94 per cent recovery (average of eight determinations); 0.90 mg., 93 per cent (three determinations); 0.45 mg., 95 per cent (eight determinations); 0.225 mg., 96 per cent (four determinations); 0.113 mg., 94 per cent (four determinations); and 0.068 mg., 93 per cent (four determinations). The results on quantities of lactic acid as small as 0.1 mg. are, therefore, as accurate as when larger amounts are analyzed. The

sample of zinc lactate used was apparently not quite pure, since 95 per cent instead of the usual 97 to 98 per cent of the theory was obtained by the macroprocedure.   The degree of reproducibility of results with blood is illustrated by the following figures—with blood lactic acid expressed in mg. per cent—which represent results of analyses of filtrates (precipitated in quadruplicate by $HgCl_2$-HCl and copper-lime) from four samples of rabbit blood: 16, 16, 16, 17; 30, 30, 31, 31; 65, 64, 65, 65; 145, 147, 146, 149.

Pyruvic acid is determinable (after reduction) in small amounts (0.4 to 0.04 mg.) by the method with the same accuracy as previously reported for larger amounts (5).

In the analysis of small amounts of lactate, the blank must be determined by each analyst with the apparatus and all of the reagents used.   It may be necessary to purify or to select different samples of reagents.   Some samples of copper sulfate and lime contribute high blanks.   The former may be purified by recrystallization, the latter by washing with hot water.

Dr. Margaret Kerly directed our attention to filter paper as a possible source of blank.   It was found that Schleicher and Schüll paper (No. 597, 12.5 cm. diameter), washed with hot water, yielded the equivalent of 0.1 mg. of lactic acid for each paper. By washing several times with hot water it is possible to rid the filter paper of the lactic acid-like material, but upon standing for 10 days in a desiccator ($CaCl_2$), the substance was again present. Therefore, if filter paper is used, it must be tested, or washed and dried immediately before use.

Oxalate interferes with pyruvic acid determinations, and cannot be used as anticoagulant where this substance is to be determined. Sodium fluoride may be used under such circumstances.

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THE USE OF SOMOGYI'S FILTRATE TO INCREASE THE SPECIFICITY OF THE GASOMETRIC BLOOD SUGAR METHOD

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Until the advent of Benedict's (1925) peculiarly specific copper reagent for blood sugar all routinely used methods agreed in determining an average sugar content of about 100 to 110 mg. per cent in normal human blood drawn during the postabsorption period. Benedict's reagent decreased this by about 20 mg. per cent, and Benedict (1928), confirmed by Van Slyke and Hawkins (1929), showed that the decrease was in the non-fermentable fraction of reducing substances. Whereas of the "sugar" determined in blood by the older methods 20 or 30 mg. per 100 cc. of blood were due to reduction of the reagents by substances which could not be fermented by yeast under conditions that completely fermented glucose, Benedict's method reduced this non-specific reduction to an amount equivalent to less than 10 mg. of glucose. Folin (1928, 1929) has devised a ferricyanide reagent which gives results similar to those of Benedict's reagent. Somogyi (1930) has shown that if the blood proteins are precipitated with zinc sulfate instead of tungstic acid the non-sugar reducing substances determinable by both copper and ferricyanide reagents, including those not so specific as Benedict's (1925, 1926) and Folin's (1928), are to a large extent removed.

The gasometric ferricyanide method of Van Slyke and Hawkins (1928), operated on the Folin-Wu (1919) tungstic acid blood filtrate, was shown by its authors (1929) to belong to the older group of less specific methods. It indicated about 20 mg. per 100 cc. more blood sugar than the Benedict method, and of the mate-

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* Hilda Stitch Stroock Fellow.

rial measured as sugar 20 to 30 mg. per 100 cc. were due to non-fermentable reducing substances.

In the present paper, however, it will be shown that when the gasometric method is applied to Somogyi's zinc sulfate blood filtrate it yields results of the same type as the recent Benedict and Folin procedures. The total sugar averages about 25 mg. per 100 cc. less than when the tungstic acid filtrate is used, and the non-fermentable reducing substance is diminished to an average glucose equivalent of 12 mg. per 100 cc.

The presence of zinc in the filtrate increases by 5 per cent the amount of ferricyanide reduced per mg. of glucose, so that, for use

TABLE I

Effect of Zinc on Reduction of Ferricyanide by Glucose

Zinc variable, glucose constant at 144 mg. per liter, equivalent to 144 mg per 100 cc blood		Zinc constant at 230 mg per liter solution; glucose variable	
oncentration of zinc in sugar solution	Increase of reduction above reduction in absence of zinc	Glucose concentration	Increase of reduction above reduction in absence of zinc
mg Zn per l	*per cent*	*mg per l.*	*per cent*
115	3 1	62 6	7 2
230	5 8	125 3	3 7
345	7 0	197 9	5 0
575	6 9	250 6	4 3
690	6 6		
1380	6 2	62 9	6 5
		125 8	3 5
		188.7	4 8
		251 6	4 2

with the Somogyi filtrates, it is necessary to revise the Van Slyke-Hawkins (1928) tables of gasometric factors by multiplying the latter by 0.95.

EXPERIMENTAL

Effect of the Zinc in Somogyi Blood Filtrates on Amount of Ferricyanide Reduced per Mg. of Glucose—The zinc content of Somogyi filtrates was gravimetrically determined. The zinc, after precipitation as ZnS, was ignited to constant weight and determined as ZnO. Preliminary determinations on a known amount of Zn by this method yielded 100 per cent recovery. On two occasions

TABLE II

*Factors for Gasometric Blood Sugar Analyses on Somogyi Filtrates**

The N_2 pressure fall, $p_0 - p_1$, multiplied by the proper factor gives blood sugar in mg. per 100 cc.

Temperature	Factors	
	Macromethod	Micromethod
°C.		
10	1 433	4 15
11	1.428	3
12	1 423	2
13	1 418	1
14	1 413	0
15	1 408	4 08
16	1 403	7
17	1 398	6
18	1 394	4
19	1 389	2
20	1 384	1
21	1 379	0
22	1 373	3 98
23	1 370	7
24	1 365	5
25	1 361	4
26	1 357	3
27	1 352	2
28	1 347	1
29	1 343	3 89
30	1 339	8
31	1 334	7
32	1 330	5
33	1 325	4
34	1 321	3

* These factors are to be used in place of those for blood analyses with Folin-Wu filtrates in Table II of Van Slyke and Hawkins (1928) and Table 42, p. 411, of Peters and Van Slyke (1932).

Somogyi blood filtrates were pooled from four different sources and the zinc content analyzed as follows: (1) 22.6 mg. per cent, (2) 23.7 mg. per cent.

Varying concentrations of zinc were added to known glucose solutions and the amounts of ferricyanide reduced were determined

TABLE III

Results with Subjects with Normal Blood Sugar

Subject No	Blood sugar found, mg per 100 cc.					Difference, Folin-Wu minus Somogyi
	Folin-Wu tungstic acid filtrates			Somogyi zinc sulfate filtrates		
	Total sugar	Fermenta-ble	Non-fer-mentable*	Total	Non-fer-mentable	Total sugar
1	118	81	37	96	15	22
2	109	73	36	84	11	25
3	110	67	43	78	11	32
4	101	69	32	79	10	22
5	104			74		30
6	107			79		28
7	107	69	38	85	16	22
8	124	83	41	100	17	24
9	109	78	31	86	8	23
10	112	76	36	86	10	26
11	109	83	26	90	7	19
Average ..	110	75	36	85	12	25

* Calculated as the difference between total sugar and Folin-Wu filtrate fermentable sugar.

TABLE IV

Results with Pathological Bloods

Age of subject	Diagnosis	Blood sugar found, mg per 100 cc.					Difference, Folin-Wu minus Somogyi
		Folin-Wu tungstic acid filtrates			Somogyi zinc sulfate filtrates		
		Total sugar	Fer-menta-ble	Non-fer-menta-ble, by differ-ence	Total	Non-fer-menta-ble	Total sugar
yrs.							
35	Diabetes	213	184	29	191	7	22
23	"	412	375	37	403	18	9
24	"	215	184	31	202	18	13
55	Polycythemia vera	166			121		45

gasometrically. The method of Van Slyke and Hawkins was followed throughout, except that the blanks contained the same

amount of zinc salts as the glucose solutions.   Anhydrous glucose was used; in the absence of zinc salts duplicate analyses yielded 99.6 and 99.8 per cent recovery.   The per cent increase in Table I indicates the extent by which the ferricyanide reduction found exceeded that calculated for the glucose present by the factors in Table II of Van Slyke and Hawkins (1928).

Comparison of Sugar Values Determined in Folin-Wu and Somogyi Filtrates—Sugar determinations were carried out on a series of bloods, separate portions of each blood being deproteinized by the two methods.   The Van Slyke-Hawkins (1928) macrotechnique was used in analyses of both types of filtrates, but in calculating the results obtained with the Somogyi filtrate the factors of Table II in this paper were used.   The fermentable sugar in the Folin-Wu filtrate was also determined by the procedure of Van Slyke and Hawkins (1929).   Fermentable sugar in the Somogyi filtrate was not determined, because it is uncertain whether the activity of yeast is impaired by the amounts of zinc present.   However, since the fermentation appears to be practically specific in the blood for glucose, the fermentable sugar value is presumably independent of the technique of deproteinization.

The results are given in Tables III and IV.   An unusually high difference, 45 mg., between results from the two filtrates was obtained in the case of polycythemia vera.   Presumably this high difference is due to the presence of unusually large amounts of intracellular non-glucose reducing substances, such as glutathione, which come through into the Folin-Wu filtrate, but are precipitated by the Somogyi technique.

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THE EFFECT OF CHOLESTEROL INGESTION ON TISSUE LIPIDS OF RATS*

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A review of the literature (1, 2) dealing with the feeding of cholesterol-containing diets shows that practically all experimental work has been limited to the rabbit. The most outstanding findings in these animals have been the production of arteriosclerosis, the deposition of fat in the liver, and the appearance of anisotropic substances which were presumably cholesterol esters. In the many papers presented in the literature, conclusions are based on the histological findings rather than on chemical analyses.

Page and Menschick (3) have presented the most complete chemical analyses of the tissues of control and cholesterol-fed rabbits which are available. These workers studied the cholesterol metabolism and demonstrated that ingested cholesterol was partially excreted or destroyed and a portion was deposited throughout the body with the exception of the brain.

The first attempt to study the fate of ingested cholesterol chemically in omnivorous animals was carried out by Yuasa (2) and Schönheimer and Yuasa (4). In a few experimental animals chemical analysis showed that the total cholesterol was increased in the livers of mice, cats, and rats fed a cholesterol-containing diet. The changes noted were small, particularly in the case of the rat. The deposition of cholesterol esters in the aortas of these animals was questionable after histological examination for anisotropic granules. As a result of these findings, Yuasa (2) believed that cholesterol metabolism in herbivorous and omnivorous animals is similar and differs only in degree.

Recently there appeared a number of reports dealing with the effect of cholesterol feeding on the lipid liver constituents in the rat. Blatherwick and his associates (5) first reported the production of fatty livers after feeding diets rich in whole dried liver to rats. No explanation could be given for the cause of this excessive fat deposition. These same workers (6) have reported that this fatty infiltration could be produced by feeding a diet containing 1.0 per cent cholesterol. The blood of the rats having fatty livers had a normal lipid content. After supplementing a control diet with 1.0 per cent cholesterol, Okey (7) noted that the cholesterol ester and total lipid concentrations rose markedly, but the lecithin values were not changed. The livers of the cholesterol-fed group were heavier than those of the control animals. Best and Ridout (8) succeeded in obtaining fatty livers in rats after feeding 100 mg. of cholesterol daily. They were able to prevent the deposition of excessive amounts of fat in the liver when choline or betaine was included in the diet.

The present investigation was begun primarily to produce arteriosclerosis in normal and partially nephrectomized rats by feeding a diet rich in cholesterol. Upon observing the unusual fatty livers in these animals, chemical analyses of the liver, kidney, blood, brain, and heart were carried out.

Experimental Procedure

The animals used in these experiments were raised in this laboratory. They were maintained on a prepared stock diet until the experimental diet was given. The majority of the animals were placed on the experimental diet at about the age of 60 days. A maximum of six rats were kept in a large double bottom cage and were allowed access to food and water up to the time of death. No record of food intake was attempted. The diets and the analyses of several of these food mixtures are given in Table I. The cholesterol (Wilson)[1] was thoroughly mixed with the other food. The rats were either killed by a blow on the neck, or, if blood was desired, the aorta was exposed under ether anesthesia and as much blood as possible was drawn with a needle and syringe. As a rule, it was possible to obtain 3 cc. of plasma for analysis. The tissues were carefully removed and the whole organ or aliquot

[1] The cholesterol was prepared by The Wilson Laboratories, Chicago.

portions were immediately prepared for extraction by the method of Osato and Heki (9). Animals were partially nephrectomized according to the directions given by Chanutin and Ferris (10).

The oxidation method of Bloor (11) with modifications by Okey (12) and Yasuda (13) was utilized for the determination of the free and ester cholesterol and total lipids. A serious source of error in this method has always been the procedure used in saponification. Bloor, in a private communication, recommended the

TABLE I

Composition of Diets

	Stock diet*	Control	Cholesterol-control	High fat-cholesterol	High carbohydrate-cholesterol
	per cent	per cent	per cent	per cent	per cent
Dried extracted beef . .		20	20	10	10
Starch (corn)		53	50 5		75 5
Lard		13	13.0	75 5	
Cod liver oil		5 0	5 0	4 0	4 0
Yeast . ..		5 0	5 0	4 0	4 0
Salt mixture (Osborne and Mendel)†.		4 0	4 0	4 0	4 0
Cholesterol			2 5	2 5	2 5
Total . .		100	100	100	100
Analysis for					
Total lipid	7 5	17 4	20 3		
" cholesterol... . ..	0 34	0 15	2 5		

* A prepared dog food mixture (Bal Ra) sold by Valentine Meat Juice Company, Richmond.

† Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **37**, 572 (1919).

use of concentrated alkali instead of sodium ethylate. The following procedure was found to yield satisfactory results: The aliquot of the alcohol-ether extract which was brought to 40 cc. was treated with 3 drops (0.16 cc.) of 40 per cent NaOH, evaporation was carried to a point where alcohol could no longer be detected (30 minutes), 0.5 to 1.0 cc. of water was immediately added, and a few drops of dilute H_2SO_4 were added until the mixture was neutral to litmus. The extraction with petroleum ether was then carried out. This simple method of saponification was

found to give recoveries of about 95 per cent with purified cholesterol palmitate alone and with tissues plus the ester.

Due to the fact that the weight of the liver varies considerably in normal animals, an effort was made to study the relationship between liver weight and a standard measurement such as weight or surface area of the rat. The ratio of liver weight to surface area × 100 proved to be satisfactory in determining the relative changes in the weight of the liver. The surface area was determined by the formula proposed by Lee (14).

Results

In Table II the analytical results are summarized for animals on stock and control diets and for normal and partially nephrectomized rats on a cholesterol-control diet.

The concentrations of total lipid of the liver and of the free cholesterol and total lipid of the blood plasma were greater in the control group than in the animals on the stock diet. These were the only differences that were noted between the animals on the stock diet and the control groups and are probably manifestations of the sensitiveness of these tissues to small differences in the fat content of the diet. The stock and control diets are low in cholesterol and contained 7.5 and 17.4 per cent total lipid, respectively. The analyses of the kidney, heart, and brain of these animals would indicate that cholesterol esters are either absent or present in traces. Extremely constant values were obtained for the free cholesterol and total lipid values in the brains of the animals on the stock diet. The lecithin concentration of the liver is slightly higher than that of the kidney. There is practically no difference in the concentration of the lecithin of the kidneys of the animals on the stock diet and that of the control animals. The average weight of the liver in these animals was about the same.

The analytical results for various tissues after a diet containing 2.5 per cent cholesterol is fed to normal rats for varying periods are given in Table II. Most of these animals were begun on the experimental diet at about 60 days of age, having previously been fed a stock ration. The analyses of kidney, blood, heart, and brain of these animals, fed on this cholesterol-rich diet for a long period of time, showed no appreciable differences in the lipid chemistry from those of the control group. The liver is the only organ show-

ing changes in the lipid chemistry. The greatest absolute change noted after cholesterol ingestion is seen in the concentration of the cholesterol esters. The average values for the free and ester cholesterol and total lipid concentrations are progressively increased with the time of feeding. Despite these changes in the lipid chemistry of the liver, the lecithin values remain normal. The weight of the fatty liver is increased above normal as evidenced by the values obtained for the liver weight to surface area which confirms Okey's (7) findings.

At autopsy, the livers of the exsanguinated rats have a light yellow tint and are very friable. Frozen sections mounted in glycerol jelly or freshly macerated liver show the presence of cholesterol esters as typified by the presence of the maltese crosses under the polarizing microscope. None of the other tissues studied, including the aorta, showed these characteristic anisotropic granules.

The kidney, liver, and blood of partially nephrectomized animals on a cholesterol-rich diet were analyzed (Table II) to find whether a disturbance in kidney function with its accompanying syndrome of hyposthenuria, polyuria, hypertension, and nitrogen retention (10) would cause any marked change in the lipid metabolism. The hypertrophied kidney stump shows marked pathological changes accompanied by edema. There is a slight decrease in the concentration of free cholesterol and total lipid of these kidneys. The free cholesterol, cholesterol esters, and total lipid concentrations in the livers of these rats are all increased. The free and ester cholesterol and total lipid concentrations of the blood are variable but unquestionably increased. It was not possible to correlate the higher values with the extent of kidney damage or hypertension.

In Table III are presented the results for rats fed a cholesterol-control diet for a relatively long time and then given cholesterol-containing diets rich in either fat or carbohydrate. Within a short time, the livers of rats fed high fat-cholesterol rations presented an interesting picture of a markedly lowered free and ester cholesterol concentration and an increased deposition of fat. On the other hand, the rats fed high carbohydrate-cholesterol rations showed an increase in the cholesterol esters together with a marked increase in the total lipid concentration. There is practically no difference in the weight of the liver in these two groups.

TABLE II[*]

Distribution of Cholesterol, Total Lipid, and Lecithin in Rat Tissues

The figures in parentheses represent the number of animals.

Diet		Cholesterol diet (days)	Age at death (days)	Liver Cholesterol Free (per cent)	Liver Cholesterol Ester (per cent)	Liver Total lipid (per cent)	Liver Lecithin (per cent)	Liver weight/Surface area × 100	Kidney Cholesterol Free (per cent)	Kidney Cholesterol Ester (per cent)	Kidney Total lipid (per cent)
Stock	Minimum		78	0.177	.007	3.18		2.02	0.358	−0.021	3.05
	Maximum		210	0.266	0.161	3.79		2.85	0.473	0.114	3.63
	Average		151	0.230 (5)	0.054 (5)	3.55 (5)		2.34 (5)	0.425 (3)	0.050 (3)	3.36 (3)
Control	Minimum		158	0.192	0.017	4.03	3.24	2.36	0.427	0.008	3.07
	Maximum		158	0.235	0.086	4.13	3.54	2.42	0.484	0.088	3.56
	Average		158	0.217 (3)	0.046 (3)	4.09 (3)	3.36 (3)	2.39 (3)	0.457 (4)	0.043 (4)	3.37 (4)
Cholesterol-control	Minimum	2	184	0.208	0.569	5.97	2.84				
	Maximum	2	193	0.274	0.762	6.51	3.30				
	Average	2	186	0.250 (4)	0.675 (4)	6.29 (4)	3.12 (4)				
	Minimum	21	84	0.212	2.22	8.65		2.40			
	Maximum	93	293	0.538	4.49	17.20		3.82			
	Average	57	195	0.311 (9)	3.38 (9)	12.26 (9)		2.95 (7)			
	Minimum	180	253	0.274	1.52	7.37	2.44	2.41	4.13	0.015	2.66
	Maximum	303	367	0.899	8.08	26.80	3.61	4.59	5.30	0.196	3.69
	Average	269	336	0.387 (9)	4.61 (9)	16.91 (9)	2.91 (5)	3.26 (9)	4.70 (7)	0.078 (7)	3.17 (7)
Partially nephrectomized rats on cholesterol-control	Minimum	167	167†	0.180	1.12	7.03		2.04	0.215	0.001	1.61
	Maximum	287	287†	0.425	8.28	19.10		3.26	0.467	0.082	3.16
	Average	212	212†	0.334 (16)	4.69 (16)	13.37 (16)		2.56 (15)	0.360 (12)	0.039 (12)	2.61 (12)

	Kidney	Blood			Heart			Brain		
	Lecithin	Cholesterol		Total lipid	Cholesterol		Total lipid	Cholesterol		Total lipid
		Free	Ester		Free	Ester		Free	Ester	
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Minimum	2 99	0 011	0 051	0 216	0 117	0 025	0 233	1 82	0.003	7.07
Maximum	3 12	0 012	0 072	0 258	0 131	0 087	0 241	1.85	0 015	7.28
Average	3 04 (3)	0 011 (3)	0 063 (3)	0 244 (3)	0 124 (3)	0 054 (3)	0 238 (3)	1 84 (3)	0 008 (3)	7.20 (3)
Minimum	2 91	0 020	0 039	0 396						
Maximum	3 48	0 032	0 070	0 593						
Average	3 21 (4)	0 028 (4)	0 057 (4)	0 476 (4)						
Minimum	2 15	0 014	0 057	0 242	0 134	−0 020	0 216	1 72	−0 007	6 53
Maximum	2 77	0 050	0.126	0 635	0 164	0 055	0 242	2 05	0 012	7.74
Average	2 37 (3)	0 028 (6)	0 084 (6)	0 382 (6)	0 151 (4)	0 027 (4)	0 229 (4)	1 93 (4)	0 002 (4)	7.03 (4)
Minimum		0 020	0.059	0 208						
Maximum		0 070	0.180	0 953						
Average		0 041 (12)	0 114 (12)	0 534 (14)						

* Each consecutive group of readings (minimum, maximum, average) in the second portion of the table applies to the respective diet of the first section in the order listed.

†Days after operation.

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From the available data, one may assume that the liver is the organ chiefly concerned in the metabolism of cholesterol. The increase in the cholesterol esters may indicate an intermediate step in the metabolism of cholesterol. No adequate explanation can be given for the deposition of fat in the liver after feeding a diet rich in cholesterol.

TABLE III

Effect of High Fat-Cholesterol and High Carbohydrate-Cholesterol Diets on Distribution of Cholesterol and Total Lipid in Liver

The figures in parentheses represent the number of animals.

	Cholesterol-control diet	High fat-cholesterol diet	High carbohydrate-cholesterol diet	Age at death	Liver			Liver weight × 100 / Surface area
					Cholesterol		Total lipid	
					Free	Ester		
	days	days	days	days	per cent	per cent	per cent	
Minimum...	74	10		136	0.166	1 16	16 5	2 36
Maximum ..	74	17		143	0 262	1 88	27 7	2 87
Average	74	14(5)		140 (5)	0 210 (5)	1 63 (5)	22 1 (5)	2 65 (5)
Minimum	45		14	130	0 254	3 93	13 20	2 13
Maximum	45		30	146	0 417	7 89	24 30	3 52
Average	45		27(5)	143 (5)	0 308 '(5)	6 10 (5)	18 74 (5)	2 89 (5)

SUMMARY

Analyses for the free cholesterol, cholesterol esters, and total lipid are presented for the liver, kidney, heart, brain, and blood of rats fed stock and control diets poor in cholesterol. Values for the cholesterol esters are extremely small in the tissues studied.

The ingestion of a cholesterol-control diet containing 2.5 per cent cholesterol resulted in the production of fatty livers. The cholesterol ester and total lipid concentrations were increased in the liver after a few days and the maximum values were obtained after 250 days. The free cholesterol concentration increased after the 3rd week of cholesterol ingestion. The other tissues studied showed no changes.

The kidneys of partially nephrectomized rats fed a cholesterol-control diet generally contained less free cholesterol than those of the control groups. The lipid values of the blood in these animals were increased.

Supplementing a cholesterol-containing diet with high concentrations of fat or carbohydrate has a striking effect on lipid metabolism. It can be readily seen that carbohydrates may accelerate and fat may inhibit the deposition of cholesterol in the liver of the rat. In addition, the total lipid concentration is markedly increased. Hence, the significance of a change in the total cholesterol of the liver must be interpreted only after the diet has been considered.

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ERGOTHIONEINE IN THE URINE*

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In previous work Sullivan and Hess (1) applied the Rupp-Schied (2) thiocyanate method as improved by Thiel (3) to various urines, normal and pathological, and found that a number of substances actually or potentially present in urine behave like thiocyanate in the Rupp-Schied-Thiel procedure. Among the substances tested was ergothioneine, a blood cell constituent, which, *a priori*, might occur in urine. On making an investigation, detailed presently, ergothioneine-like substances were found in urine and a certain amount of ergothioneine was actually isolated.

Ergothioneine—This compound isolated from blood by Benedict (4), Benedict, Newton, and Behre (5), and Hunter and Eagles (6, 7), was found by Newton, Benedict, and Dakin (8) to be identical with the base ergothioneine isolated from ergot by Tanret (9) and shown by Barger and Ewins (10) to be the betaine of thiohistidine. Previously no one has shown its presence in normal or pathological urines.

Proof of Presence of Ergothioneine-Like Substances in Urine—Benedict, Newton, and Behre (5) showed that ergothioneine is precipitated by silver lactate in the presence of lactic acid but is not freed from the silver compound by treatment with a 10 per cent solution of sodium chloride in 0.1 N hydrochloric acid as used in the Folin-Wu (11) method for uric acid. In this way ergothioneine can be separated from uric acid which is extracted by acid sodium chloride from the silver precipitate. This procedure was applied to urine as follows: To 3 cc. of urine, Folin-Wu's silver lactate-lactic acid mixture was added in slight excess.

The mixture was centrifuged and the supernatant liquid decanted. The insoluble residue was washed by stirring with acid-sodium chloride (10 per cent sodium chloride in 0.1 N hydrochloric acid) in 50 cc. lots, centrifuging, and decanting until the washings no longer gave a color with the uric acid reagent.

A study was then made of the optimum amount of sodium cyanide needed to bring the silver precipitate into solution and give the maximum development of color with the uric acid reagent. Finally the use of 6 cc. of 5 per cent cyanide was found best for both purposes. The validity of this statement is shown by the following experiments, Experiment A on ergothioneine-like material in urine and Experiment B with blood ergothioneine added to a solution of a number of urinary ingredients.

Experiment A—The contents of four tubes, each containing 3 cc. of the same urine, were precipitated by silver lactate, centrifuged, and washed four times with 40 cc. of the acid-sodium chloride until the washing gave no color with the Folin-Marenzi (12) reagent and alkali. Then the respective solid was treated as follows:

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Tube  1 + 0.5 cc. 5 per cent NaCN + 5 5 cc. 0.8 N NaOH
 "    2 + 1.0 "  5 "     "      "   + 5 0 "  0.8 "   "
 "    3 + 3 0 "  5 "     "      "   + 3 0 "  0.8 "   "
 "    4 + 6 0 "  5 "     "      "   + 0.0 "  0.8 "   "
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To each tube were then added 5 cc. of water and 0.5 cc. of reagent.

Tubes 1 and 2 gave no blue color. Tube 3 gave a good blue but less than Tube 4.

Experiment B—Ergothioneine from blood was added to an artificial mixture containing 1 gm. of NaCl, 2.5 gm. of urea, 0.07 gm. of creatinine, 0.01 gm. of potassium thiocyanate, 0.001 gm. of oxalic acid, and 0.15 gm. of sulfuric acid in 100 cc. of water. Four tubes, each containing 0.25 mg. of ergothioneine in 3 cc. of the mixture were treated as in Experiment A. Similar results were obtained.

Accordingly, the use of 6 cc. of the cyanide was made a routine procedure. To the silver complex dissolved in the sodium cyanide were added 1 cc. of the Folin-Marenzi reagent and 1 cc. of N sodium hydroxide. The standard solution for comparison was 0.5 mg. of ergothioneine dissolved in 6 cc. of 5 per cent sodium cyanide and treated in the same manner as the unknown.

In this way, ergothioneine-like material was estimated in eleven normal urines, thirteen urines from patients with cancer, including cancer of the lung, breast, uterus, prostate, and sarcoma of chin, lower bowels, etc., nine arthritics, and twenty other cases with a wide variety of pathological conditions. The findings are given in Table 1.

The values for cancer cases tend to run high. Since, however, three of the cases of malignancy, a cancer of the prostate, a cancer of the lung, and a lymphosarcoma of the lower bowel gave ergothioneine values of 82, 80, and 62 mg. respectively, in the 24 hour urine, the estimation of ergothioneine-like material does not seem to have any diagnostic value for malignancy. This conclusion is corroborated by the fact that high values were obtained occasionally in other conditions, as for example, arthritis.

TABLE I

Ergothioeine-Like Material in Urine

Condition	Amount in 24 hr. urine			Mean volume	Mean weight
	Mini-mum	Maxi-mum	Mean		
	mg	*mg.*	*mg*	*cc*	*mg per l*
Normal ..	77 6	154	113 4	1267	89 5
Cancer	62 0	198	135 0	911	147 1
Arthritis	50 5	215	123 7	1261	98 1
Other pathological cases .	34 0	225	90 6	1020	88 5

Since Behre and Benedict (13) in their study of blood give evidence for the presence of other material behaving like ergothioneine in their procedure, the material judged to be present in urine is called ergothioneine-like. There is evidence, however, that ergothioneine itself, at least in a small amount, is present in normal urine.

Isolation of Ergothioneine from Urine—The next step was to isolate ergothioneine from urine. This was accomplished by a modified Hunter and Eagles (7) procedure for blood followed in part by Benedict's (5) procedure. 12 liters of normal urine were brought to pH 3.5 by the addition of glacial acetic acid. Basic lead acetate (Goulard's reagent) was then added until the supernatant liquid gave a precipitate with sulfuric acid. 500 cc. of

Goulard's solution were required. The filtrate was freed from lead by hydrogen sulfide. The filtrate from lead sulfide, freed from hydrogen sulfide by passing air through it, was treated with a saturated alcoholic solution of mercuric chloride as long as a precipitate formed. The mercury precipitate was decomposed by hydrogen sulfide and thoroughly washed. The solution, made to 0.5 N acid with sulfuric acid, was precipitated with phosphotungstic acid until precipitation ceased. A pink precipitate formed which was allowed to stand overnight at 5°. Then the phosphotungstic precipitate was ground with an excess of barium hydroxide and filtered. The filtrate freed from barium was concentrated under reduced pressure to 30 cc. Considerable material separated out. The filtrate from this precipitate was treated with silver lactate in slight excess and centrifuged. The silver precipitate was then washed by stirring with a solution of 10 per cent sodium chloride in 0.1 N hydrochloric acid in 50 cc. lots to a total of 600 cc. until the supernatant liquid gave a negative reaction with the Folin-Marenzi uric acid reagent and sodium hydroxide. The silver precipitate was then washed into a beaker containing 30 cc. of hot 0.5 N hydrochloric acid and the mixture was boiled for 5 minutes. The filtrate was then placed in the ice box at 5° overnight. After filtering from a precipitate the solution was concentrated to a few cc. and an equal volume of absolute alcohol was added. On standing a white compound settled out. This was filtered and dried in a desiccator over calcium chloride. The weight was 60 mg.

This material behaves like ergothioneine in that it reacts with the Folin-Marenzi uric acid reagent, gives a red color with Hunter's (14) diazo reaction for ergothioneine, and titrates like ergothioneine in Okuda's iodometric titration as used for cystine.

Analyzed for nitrogen by Kjeldahl and for sulfur by Parr bomb it gave nitrogen 15.26 per cent, sulfur 11.95 per cent. The calculated values for ergothioneine hydrochloride ($C_9H_{15}N_3SO_2HCl$) are N 15.81 per cent, sulfur 12.07 per cent. The free base obtained by the method of Benedict, Newton, and Behre melted at 261° and decomposed between 285–290°.

In previous work (15) ergothioneine from blood was found to give a titer in Okuda's (16) iodometric method for cystine. In the present work, the free base reduced by zinc and hydrochloric

acid, 2.5 mg., gave a titer of 0.52 cc. of 0.001 M KIO_3 or 0.45 cc. of 0.001 M KIO_3 for the hydrochloride.   The material isolated from urine, similarly treated gave a titer of 0.45 cc. for 2.5 mg., the theoretical for ergothioneine hydrochloride.

Analysis and reactions indicate the presence of ergothioneine in urine.   The amount isolated, 5 mg. per liter, is small but this may be due to the wasteful procedure involved.   The procedure is by no means quantitative and was used merely to show the actual presence of ergothioneine.   Colorimetrically, there were found about 90 mg. per liter of ergothioneine-like material in normal urine.

5 liters of normal urine were then tested for ergothioneine by the red oxide of copper precipitation method of Williamson and Meldrum (17).   Colorimetrically the urine showed 446.5 mg. in the 5 liters.   However, in concentrating the urine under reduced pressure to 550 cc. preparatory to treatment with the red oxide a loss of about 34 per cent was encountered in material reacting like ergothioneine in treatment with silver lactate and colorimetric comparison with blood ergothioneine.   With the copper oxide procedure, the ergothioneine isolated was again of the order of magnitude as given above, 5 to 6 mg. per liter.   These figures are undoubtedly minimal figures.   Assuming that the isolation procedures are satisfactory when applied to urine, it would seem that most of the ergothioneine-like material found by us in normal urine is not ergothioneine.   Its nature is being further investigated.

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CRYSTALLINE EGG ALBUMIN

THE HYDROLYSIS OF CRYSTALLINE EGG ALBUMIN BY PEPSIN, PAPAIN-HYDROCYANIC ACID, AND PANCREATIC PROTEINASE AND THE SUBSEQUENT ACTION OF SOME OTHER ENZYMES ON THE HYDROLYSIS PRODUCTS PRODUCED BY THESE ENZYMES*

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The investigation of the action of enzymes on egg albumin is of 4-fold importance. First, albumin is one of the most important proteins in abundance in the egg, consequently the changes—necessarily enzymatic—which occur in this protein during the embryonic development of the chick are of considerable interest. Secondly, the general knowledge of the structure of proteins leads to the conclusion that at present one of the best methods of investigation of their structure is the study of the action of homogeneous enzymes on them. Thirdly, since the action of homogeneous enzymes on pure proteins of high molecular weight had not been studied previously, it was of interest to know what information such a study would yield. Fourthly, egg albumin is a common protein of the daily diet and often plays an important rôle in the allergic reactions of certain individuals, particularly children. It is quite possible that an investigation of the nature of enzyme action on this protein and a study of the products formed, which the author plans to continue in the immediate future, will aid in the elucidation of this complex biological phenomenon.

Previous investigations of the extent of enzyme action on egg albumin and of the nature of the products formed are for the most part invalid for two reasons: namely, the protein used in most cases was not crystalline egg albumin (*i.e.*, pure egg albumin)

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while the enzyme preparations used were, as is now recognized, mixtures of several enzymes.   Umber (1) in 1898 treated crystalline egg albumin with pepsin and separated the digestion products by fractional precipitation with ammonium sulfate.   Later Zunz (2) made a similar study using zinc sulfate as the fractionating agent.   Herzog and Margolis (3) reported that pepsin liberated 50 per cent of the total nitrogen as amino nitrogen while trypsin liberated 70 per cent.   Henriques and Gjaldbaek (4), using the formol titration method, found that pepsin set free 38 per cent and trypsin, 63 per cent of the total nitrogen present as amino nitrogen. Rudd (5) hydrolyzed egg albumin with pepsin and separated the hydrolysis products by means of ammonium sulfate and alcohol into four fractions.   McFarlane, Dunbar, Borsook, and Wasteneys (6) found that most of the products of peptic hydrolysis of egg albumin arose directly from the protein molecule and underwent no further change, while the proteose representing about 15 per cent of the total nitrogen was subject to a slow secondary hydrolysis in which simpler products were formed.   Dauphinee and Hunter (7) have made the interesting observation that after tryptic digestion 80 per cent of the arginine could be determined by arginase.   This has been confirmed by Torbet and Bradley (8) who preceded the tryptic digestion by peptic digestion.   Desai (9) has separated the products of peptic hydrolysis of egg albumin by fractional dialysis and has determined the total amino, peptide, and non-amino nitrogen in the various fractions which in most cases showed distinct difference.   The action of papain on proteins has been extensively studied by Willstätter, Grassmann, and Ambros (10) but they did not use crystalline egg albumin.   The most recent investigation is a preliminary report by Svedberg and Erikson (11).

Below the author gives the methods used with only a part of the experimental detail, since it would require too much space to give it all, and discusses the results obtained when pepsin, papain-hydrocyanic acid, and the proteinase of pancreas act on pure crystalline egg albumin as well as the results obtained when the primary split-products are subjected to the further action of some other enzymes, namely pepsin at pH 2 to 2.5, papain-hydrocyanic acid at pH 5, protaminase, aminopolypeptidase, carboxypolypeptidase, and dipeptidase, all at pH 7.5.   The carboxypolypeptidase and dipeptidase were not isolated and used individually but the manner

of determining their action is discussed in the experimental part. A summary of the results is given in Tables I to III. For several reasons it is best to separate the experimental section into two parts. The individual experiments reported in Parts A and B are not to be confused with the summarized experiments reported in Tables I to III. The numbers do not necessarily correspond.

EXPERIMENTAL

Part A. Crystalline Albumin—The crystalline albumin used was prepared according to the method of Sörensen and Høyrup (12, 13). Due to the particle size of the dried material enzyme action was very slow on such material, so it was necessary to grind it in a ball mill with water until it formed a fine suspension which settled very slowly.

A second sample of albumin was preserved with toluene and dialyzed free of inorganic salts. It was then kept as approximately a 3 per cent solution, preserved with toluene in an ice box at 0°.

Proteinase of Pancreas—Proteinase was prepared according to the method of Waldschmidt-Leitz and Purr (14) in which a glycerol extract of dried pancreas was diluted with an equal volume of water, activated with kinase if necessary, and the erepsin removed by adsorption with aluminum hydroxide $C\gamma$ at pH 4, while the carboxypolypeptidase and protaminase were subsequently removed at pH 7 by aluminum hydroxide A according to a modified method of Waldschmidt-Leitz and Schäffner (15). The solution ranged in unitage, measured by the method of the above authors, from 0.35 to 0.5 trypsin unit per cc. Before use the solutions were tested for the presence of protaminase, carboxypolypeptidase, and erepsin.

Pepsin and Papain—The pepsin and papain were purchased from Merck, the papain preparation being Merck's papayotin 1:80.

Aminopolypeptidase—This enzyme was prepared from a glycerol extract of intestinal mucosa (swine) according to the method of Waldschmidt-Leitz and Schäffner (16). The glycerol extract was diluted with an equal volume of water adjusted to pH 4, cooled in ice, centrifuged to remove the precipitate formed, and then absorbed several times with small amounts of ferric hydroxide. The solution which then contained only aminopolypeptidase was

neutralized to pH 7.   The strength of the enzyme solutions used in this investigation was always the following or greater: 2 cc. of enzyme solution and 5 cc. of 0.005 M leucylglycylglycine solution (0.001 M) placed in a thermostat at 30° for 1 hour caused an increase in carboxyl groups equivalent to 0.70 cc. of 0.2 N alkali or more.   The enzyme solution was free of carboxypolypeptidase and dipeptidase.

Protaminase—At present it is not possible to prepare a solution in which protaminase alone is present but it is quite easy to prepare a solution according to the method of Waldschmidt-Leitz, Ziegler, Schäffner, and Weil (17) in which only protaminase and inactive proteinase are present.   A glycerol extract of dried fresh pancreas of pigs was used and was adsorbed first at pH 4 and then at pH 7 with aluminum hydroxide $C\gamma$, to remove first the erepsin and later the carboxypolypeptidase.   The absence of these enzymes and the inactivity of proteinase was always proved by experiment.

Carboxypolypeptidase—The extent of carboxypolypeptidase action in this series of experiments was determined by the amount of hydrolysis caused by pancreatic extract after complete removal of erepsin.   The method used here is justifiable since the extent of hydrolysis by proteinase and protaminase can be accurately determined and after the removal of erepsin (aminopolypeptidase and dipeptidase) no other proteolytic enzyme is known to exist in an extract of pig pancreas.

Dipeptidase—A procedure similar to that used for carboxypolypeptidase was adopted for dipeptidase.

Substrates—The following substrates were used to establish the presence or absence of the various enzymes and to determine the strength of the solutions used.   A casein solution containing 6 gm. of casein and 6 cc. of N sodium hydroxide in a total volume of 100 cc. was used as the substrate for proteinase.   For protaminase a solution prepared according to an unpublished method of Waldschmidt-Leitz, Schäffner, and Weil was used.   The solution was prepared as follows: 10 gm. of clupein sulfate in 200 cc. of exactly 10 per cent by volume sulfuric acid were immersed in a vigorously boiling water bath for exactly 2.5 hours, cooled immediately, and concentrated sodium hydroxide solution was added with cooling until the pH of the solution was 8.   The ratio of total nitrogen to amino nitrogen should be 6.90.   On this substrate proteinase is

inactive and protaminase is very active. The substrate for carboxypolypeptidase was a solution containing 0.515 gm. of chloroacetyl-*l*-tyrosine neutralized to pH 7.4 with brom-thymol blue plus 16 cc. of M/15 disodium phosphate and 4 cc. of M/15 monosodium phosphate in a total volume of 50 cc. The standard substrate for amino polypeptidase was prepared as follows: 4.90 gm. of *dl*-leucylglycylglycine were dissolved in 40 cc. of a solution of ammonium hydroxide-ammonium chloride (1:1 mixture of N solutions) and a little water. 5 cc. of N acetic acid were added and the total volume made up to 100 cc. The resulting pH of the solution was 8. The substrate for dipeptidase was made in exactly the same manner by using 3.764 gm. of *dl*-leucylglycine. 5 cc. of the above substrate with 2 cc. of the enzyme solutions were used in determinations. The time was 1 hour (except for proteinase which was 20 minutes) when determining the strength of the solution for use but 18 to 24 hours when determining the absence of enzymes from the solutions to be used.

The papain solution was prepared and tested in the following manner: 500 mg. of papayotin powder (Merck) were suspended in 15 cc. of water to which were added 4 cc. of 0.5 M phosphate buffer of pH 7, 20 cc. of 5 per cent potassium cyanide solution, and methyl red indicator. N hydrochloric acid was then added until the pH of the solution was approximately 5 as shown by the indicator present. The volume was made up to 50 cc. and the solution was allowed to activate at 30° in a thermostat for 2 hours. The strength of the filtered enzyme solution was then tested as follows: 2 cc. of enzyme solution, 2.5 cc. of 0.1 M disodium citrate (pH 5), and 1 gm. of gelatin (12.5 cc. of an 8 per cent solution) were mixed and the volume made up to 25 cc. A 10 cc. blank determination was made immediately in the Van Slyke apparatus. After 1 hour another 10 cc. determination was made. The amount of hydrolysis was usually sufficient to furnish enough amino groups to give a volume of 2 to 3 cc. of gas in the Van Slyke determination.

To illustrate the manner in which the investigation was carried out, two typical experiments have been chosen from approximately 90 similar ones which could be given if such details were required. In some cases it was necessary to make blank determinations and to correct where the subsequent enzyme showed activity on a heated and filtered solution of the preceding enzyme.

Experiment I—An albumin suspension for pancreatic proteinase digestion was obtained by denaturation for 30 minutes of an albumin solution in a boiling water bath.   76 cc. of this suspension, containing 338.8 mg. of nitrogen, were mixed with 200 cc. of pancreatic proteinase solution (60 trypsin units), adjusted to pH 7.5, and the volume made up to 300 cc.   The digestion was carried out at 30° in a thermostat.   1 cc. contains 7.333 mg. of albumin or 1.129 mg. of nitrogen.

November 4, 10 cc. blank in Van Slyke apparatus 2.60 cc. (21°, 746 mm.); 8 hours later, 10 cc. of digestion mixture in Van Slyke apparatus 5.80 cc. (21°, 745 mm.); 24 hours later, 10 cc. of digestion mixture 7.20 cc. (19°, 739 mm.); 72 hours later, 10 cc. of digestion mixture 7.60 cc. (20°, 742 mm.); 96 hours later, 10 cc. of digestion mixture 7.60 cc. (21°, 745 mm.).   At this point fresh enzyme was added to the above digestion mixture.   Blank on 10 cc. of the digest and 10 cc. of fresh enzyme 8.40 cc. (21°, 745 mm.); after 24 hours action of the fresh enzyme, 10 cc. of digestion mixture and 10 cc. of the enzyme gave 8.45 cc. (20°, 748 mm.).

There was no further digestion by fresh proteinase.   The calculated amino nitrogen increase was 83.2 mg. or 24.6 per cent of the total nitrogen; when calculated for 200 mg. of albumin in terms of 0.2 N alkali the value was 2.70 cc.

The titration of an aliquot of the above solution in 90 per cent alcohol with 0.2 N alkali, thymolphthalein being used as the indicator, gave the following results.

November 4, 30 cc. blank titration 3.65 cc. of 0.2 N KOH; 120 hours later, 30 cc. of experimental titration 6.52 cc. of 0.2 N KOH.   The increase was 2.87 cc. for 220 mg. of albumin or 2.61 cc. for 200 mg.

Experiment II—The action of erepsin following the action of proteinase was as follows: 100 cc. of the digest from Experiment I and 100 cc. of erepsin were digested at pH 7.5 and 30°.   Total volume 200 cc., total albumin 733.3 mg., total nitrogen 112.9 mg.

November 21, 10 cc. blank in Van Slyke apparatus 5.80 cc. (21°, 740 mm.); 96 hours later, 10 cc. of digestion mixture in Van Slyke apparatus 10.78 cc. (21°, 737 mm.); 120 hours later, 10 cc. of digestion mixture 10.70 cc. (21°, 742 mm.).   At this point fresh enzyme was added to the above digestion mixture.   Blank on 10 cc. of the digest and 5 cc. of fresh enzyme 12.13 cc. (21°, 742 mm.); after 24 hours action of the fresh enzyme, 10 cc. of digestion mixture and 5 cc. of the enzyme gave 12.15 cc. (20°, 740 mm.).

There was no further action by the crepsin. The total amino nitrogen liberated was 53.97 mg. or 47.8 per cent of the total amino nitrogen present. For 200 mg. of albumin this was equivalent to 5.14 cc. of 0.2 N alkali. The total amino nitrogen after complete digestion is the sum of the amino nitrogen present before enzyme action is begun (3.3 per cent), plus the amino nitrogen liberated by proteinase (24.5 per cent), and the amino nitrogen liberated by crepsin (47.8 per cent) which is 75.7 per cent, over 72 per cent of which is due to the action of enzymes.

A titration of an aliquot of the above in alcohol with 0.2 N alkali with thymolphthalein as indicator gave the following results.

Nov. 21, 50 cc. blank   titration =   5.77 cc. 0.2 N KOH
  "   27, 50 " experimental   "   = 10.38 "   0.2 "   "

The increase was 4.61 cc. of 0.2 N KOH and when calculated for the total digestion mixture this was equivalent to 51.64 mg. of amino nitrogen or 45.7 per cent of the total nitrogen, a value in quite good agreement with the 47.8 per cent obtained above by the Van Slyke method. Calculated for 200 mg. of albumin the equivalent was 5.03 cc. of 0.2 N alkali.

In all of these experiments the amino nitrogen was determined by the macromethod of Van Slyke (18) and the increase in carboxyl groups determined by titration in 90 per cent alcohol according to the method of Willstätter and Waldschmidt-Leitz (19), with thymolphthalein as the indicator. The results of the entire series of experiments are summarized in Table I.

Part B—It was found that either the albumin suspension or uncoagulated albumin served equally well as substrates for pepsin and that the end-results, as far as free amino nitrogen and free carboxyl groups were concerned, were always the same within a very small range of error. It was also true that by the use of large quantities of pepsin the hydrolysis could be completed in a much shorter time than when smaller amounts were used. When 2.5 gm. of pepsin were used per gm. of egg albumin, the reaction came to a point within 8 to 14 days where the further addition of pepsin produced no further increase in amino nitrogen, while if 200 mg. of pepsin were used in the beginning, it was found necessary to add more pepsin and continue the action for 30 to 40 days. More constant results were obtained by the first procedure and the time

<div align="center">TABLE I</div>

Hydrolysis by Trypsin, Pepsin, Papain, Protaminase, Carboxypolypeptidase, Aminopolypeptidase, and Erepsin

Calculation is made on the basis of 200 mg. of ash- and moisture-free crystalline egg albumin. The increase in —COOH groups was determined by titration in alcohol and the increase in amino groups by the method of Van Slyke. Both are calculated in cc. of 0.2 N acid and amino nitrogen in per cent of total nitrogen. Trypsin = pure pancreatic proteinase.

Experiment No.	Sequence of enzymes	Increase (cc 0 2 N)		Amino N of total N	Probable relationship	Peptide linkages hydrolyzed
		COOH	NH₂			
				per cent	*per cent*	
1	Trypsin	2 66	2 65	24–25	24	90
	Pepsin	0	0	0		
2	Trypsin	2 66	2 65	24–25	24	90
	Papain-HCN		2 64	24–25	24	90
	Sum		5 29	48–50	48	180
3	Trypsin	2 66	2 65	24–25	24	90
	Protaminase	0 64	0 68	6	6	20
	Aminopolypeptidase	3 16	3 12	30–31	30	105
	Sum	6 46	6 45	60–62	60	215
4	Trypsin	2 66	2 65	24–25	24	90
	Aminopolypeptidase	4 14	3 96	37 38	36	125
	Sum	6 80	6 61	61 63	60	215
5	Trypsin-protaminase-carboxypolypeptidase	6.70	6 63	61–62	60	215
6	Trypsin-protaminase	3 25	3 45	31–32	30	110
7	Trypsin	2 66	2 65	24–25	24	90
	Protaminase	0 64	0.68	6	6	20
	Erepsin	4 50	4 46	41–42	42	160
	Sum	7 80	7.79	71–73	72	270
8	Trypsin	2 66	2.65	24–25	24	90
	Erepsin	5 04	5.14	47–48	48	180
	Sum	7 70	7 79	71–73	72	270

of waiting for the termination of the hydrolysis was not so long. It may be well to mention that the action of pepsin is extremely rapid at the beginning so that a blank determination made after 5 to 10 minutes is no longer a blank.

Three experiments were selected as typical of the many performed in this investigation and are reported in detail below.

Experiment I. Pepsin Digestion of Albumin Suspension—50 cc. of albumin suspension containing 1.165 gm. of albumin were treated with 2.5 gm. of pepsin (as a 10 per cent solution), the pH was adjusted to 2, and the total volume made up to 80 cc. 1 cc. contained 14.56 mg. of albumin and 2.241 mg. of nitrogen. The mixture was preserved with toluene and placed in a thermostat at 30 degrees.

October 27, 5 cc. blank in Van Slyke apparatus 8.05 cc. (19°, 741 mm.); 264 hours later, 5 cc. of digestion mixture in Van Slyke apparatus 12.90 cc. (19°, 743 mm.); 288 hours later, 5 cc. of digestion mixture 13.20 cc. (19°, 742 mm.); 312 hours later, 5 cc. of digestion mixture 13.15 cc. (19°, 741 mm.). At this point fresh enzyme was added to the above digestion mixture. Blank on 5 cc. of the digest and 5 cc. of fresh enzyme 14.85 cc. (19°, 741 mm.); after 24 hours action of the fresh enzyme, 5 cc. of digestion mixture and 5 cc. of the enzyme gave 14.85 cc. (20°, 742 mm.).

There was no further digestion by addition of more pepsin. The calculated amino nitrogen liberated by pepsin was 45.91 mg. or 25.6 per cent of the total nitrogen.

A titration experiment exactly as above was carried out and a 10 cc. blank titration in 90 per cent alcohol, with thymolphthalein, required 17.02 cc. of 0.2 N alkali, while 10 cc. of the digestion mixture 14 days later, after the amino nitrogen by the Van Slyke method showed no further increase, required 19.20 cc. of 0.2 N alkali. Calculated in terms of amino nitrogen the increase was 48.83 mg. or 23.8 per cent of the total nitrogen.

Experiment II—4 cc. of the pepsin digest from Experiment I were heated and filtered and 35 cc. of protaminase solution added. pH 7.5, total volume 80 cc.; 1 cc. contained 7.25 mg. of albumin or 1.1205 mg. of nitrogen.

November 17, 5 cc. blank in Van Slyke apparatus 6.11 cc. (21°, 745 mm.); 8 hours later, 5 cc. of digestion mixture in Van Slyke apparatus 7.10 cc. (21°, 746 mm.); 24 hours later, 5 cc. of digestion mixture 7.66 cc. (22°, 745 mm.); 48 hours later, 5 cc. of digestion mixture 7.65 cc. (21°, 746 mm.). At this

point fresh enzyme was added to the above digestion mixture.   Blank on 5 cc. of the digest and 5 cc. of fresh enzyme 8.13 cc. (22°, 746 mm.); after 24 hours action of the fresh enzyme, 5 cc. of digestion mixture and 5 cc. of the enzyme gave 8.15 cc. (21°, 745 mm.).

There was no further increase with fresh enzyme.   The total increase in volume was 1.55 cc.   The volume of gas produced by the action of the same amount of enzyme on an equivalent amount of pepsin to that present in the above solution was 0.84 cc.   The amount of gas from the amino groups set free was then 0.35 cc., which is equivalent to 6.27 mg. of nitrogen or 7.0 per cent of the total nitrogen as amino nitrogen.   The experiment was repeated twice with approximately the same results and in a titration experiment with 250 mg. of albumin in each titration the value 6.93 per cent was obtained.

Experiment III—20 cc. of the digestion mixture from Experiment II containing 145 mg. of albumin and 22.41 mg. of nitrogen were treated with 48 cc. of erepsin; pH 7.5 and total volume made up to 70 cc.

November 25, 10 cc. blank in Van Slyke apparatus 8.45 cc. (20°, 747 mm.); 24 hours later, 10 cc. of digestion mixture in Van Slyke apparatus 14.92 cc. (20°, 742 mm.); 48 hours later, 10 cc. of digestion mixture 14.96 cc. (21°, 750 mm.).   At this point fresh enzyme was added to the above digestion mixture.   Blank on 10 cc. of the digest and 5 cc. of fresh enzyme 16.23 cc. (21°, 750 mm.); after 24 hours action of the fresh enzyme, 10 cc. of digestion mixture, and 5 cc. of the enzyme gave 16.25 cc. (20°, 747 mm.).

There was no further increase and when the calculation was made and corrected for the action of erepsin on pepsin itself, 8.38 mg. of amino nitrogen were found' to be liberated from 145 mg. of albumin, which is equivalent to 37.4 per cent of the total nitrogen. The amino nitrogen set free in this series of experiments was 70.0 per cent of the total nitrogen.   There was present in the original albumin 3.4 per cent of the total nitrogen as amino nitrogen so that the total amount of amino nitrogen present at the end of digestion was 73.4 per cent of the total nitrogen.   The average value from four experiments, in which the percentage of amino nitrogen in terms of the total nitrogen was determined following acid hydrolysis with both sulfuric and hydrochloric acids, was 72.9 per cent.   The results of the experiments in which pepsin was the

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TABLE II

Hydrolysis by Pepsin, Trypsin, Papain, Protaminase, Carboxypolypeptidase,
Aminopolypeptidase, and Erepsin

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Sequence is given and the amount calculated as amino nitrogen in per cent of total nitrogen. Calculation is made on the basis of 200 mg. of ash- and moisture-free crystalline egg albumin. Increase in --COOH groups was determined by titration in alcohol and the increase in amino groups by the method of Van Slyke. Both are calculated in cc. of 0.2 N acid. Trypsin = pure pancreatic proteinase.

Experiment No.	Sequence of enzymes	Increase (cc. 0.2 N)		Amino N of total N	Probable peptide linkages hydrolyzed	Probable percentage of splitting
		COOH	NH₂			
				per cent		
1	Pepsin	2 70	2 81	25	90	24
	Trypsin	0	0	0		
2	Pepsin	2 70	2 81	25	90	24
	Papain-HCN		1.86	20	72	20
	Sum		4 67	45	162	44
3	Pepsin	2.70	2.81	25	90	24
	Protaminase	0.88	0 89	7	25	7
	Aminopolypeptidase	2 12	2 00	18	65	17
	Sum	5 70	5 70	50	180	48
4	Pepsin	2 90	2 84	25	90	24
	Protaminase + carboxy-polypeptidase	2 67	2.70	25	90	24
	Sum	5 57	5.54	50	180	48
5	Pepsin	2.70	2.81	25	90	24
	Aminopolypeptidase	2 73	2.81	25	90	24
	Sum	5 43	5.62	50	180	48
6	Pepsin	2 70	2 81	25	90	24
	Protaminase	0 88	0 89	7	25	7
	Erepsin	4.18	4.13	38	155	41
	Sum	7.76	7.83	70	270	72
7	Pepsin	2 70	2.81	25	90	24
	Erepsin	4 99	5 10	45	180	48
	Sum	7.69	7.91	70	270	72
8	Acid hydrolysis			72.80	270	72

initial enzyme used are summarized in Table II and the results of a similar series with papain are summarized in Table III.

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TABLE III

Hydrolysis by Papain, Pepsin, Trypsin, Protaminase, Aminopolypeptidase, Carboxypolypeptidase, and Erepsin

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Calculation is made on the basis of 200 mg. of ash- and moisture-free crystalline egg albumin.   The increase in —COOH groups was determined by titration in alcohol and the increase in amino groups by the method of Van Slyke, both being calculated in cc. of 0.2 N acid and the amino N in per cent of the total nitrogen and expressed in the nearest whole number. Trypsin = pure pancreatic proteinase.

Experiment No.	Sequence of enzymes	Increase (cc. 0.2 N)		Amino N of total N	Probable percentage of splitting	Peptide linkages hydrolysed
		COOH	NH₂			
				per cent		
1	Papain-HCN	5 48	5.66	51	48	180
	Pepsin	0	0	0	0	0
2	Papain-HCN	5.48	5 66	51	48	180
	Trypsin	0	0	0	0	0
3	Papain-HCN	5 48	5.66	51	48	180
	Protaminase	0	0	0	0	0
4	Papain-HCN	5 48	5.66	51	48	180
	Aminopolypeptidase		1 02	9	12	35
	Sum		6.68	60	60	215
5	Papain-HCN	5 48	5 66	51	48	180
	Carboxypolypeptidase		1.16	10	12	35
	Sum		6.82	61	60	215
6	Papain-HCN	5.48	5.66	51	48	180
	Erepsin		2 17	19	24	90
	Sum		7 83	70	72	270

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DISCUSSION

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The hydrolysis of pure egg albumin with the pure proteinase of pancreas is the first example of the study of the hydrolysis of a high molecular weight protein by this proteinase and likewise the first determination of the extent of its action.   It is also the first demonstration that liberation of free amino and free carboxyl groups from egg albumin by pancreatic proteinase is the same.

During the course of this investigation the experiment was repeated several times and the results were surprisingly constant. In each case a value between 24 and 25 per cent of the total nitrogen as amino nitrogen was found. In the titration experiments the number of carboxyl groups liberated corresponded almost exactly to the number of amino groups as can be seen from Experiment I of Part A and from Table I. This indicates that the linkages split by this enzyme were only the peptide linkages of such a nature that a carboxyl group and a primary amino group were liberated simultaneously. These results agree with the previous findings of Waldschmidt-Leitz and Simons (20) in the case of pepsin and if proline peptide linkages such as those shown by Bergmann, Zervas, and Schleich (21) to exist in gelatin, are hydrolyzed, the number is quite small and does not correspond to the amounts of proline and oxyproline which are present in albumin. Small amounts, however, might be liberated and not recognized by these methods.

The action of the proteinase was very rapid when the albumin was freshly coagulated in a fine suspension and if sufficient enzyme was used the albumin was all in solution within 5 hours and completely digested within 24 hours. When uncoagulated albumin was treated with pancreatic proteinase, the same phenomenon of very slow digestion was observed which has been often seen in the case of commercial pancreatin and egg white. In our experiments the action was very slow but after about 14 days gave the same end-results as did a similar experiment with coagulated albumin in 48 hours. The explanation of this phenomenon is unknown but as was shown by experiment the slow hydrolysis was a true enzyme action and not due to the alkali present.

In previous investigations it has been reported that after the action of trypsin, pepsin would still digest the protein split-products, but in the case of crystalline albumin, as shown in Table I, after the digestion by pancreatic proteinase there was no further action by pepsin; neither was there any action of pancreatic proteinase on the pepsin split-products of albumin. This at first might lead one to conclude that the same linkages were hydrolyzed by the two proteinases; but this was not necessarily the case as was indicated by subsequent experiments.

The recently discovered enzyme, protaminase (17), acts on the

protamines from which substances it splits off free arginine.   In the case of egg albumin it reacted neither with the denatured nor with the undenatured products to the slightest extent but following proteinase digestion it reacted rapidly with the products formed liberating almost exactly 6 per cent of the total nitrogen as amino nitrogen.   This is equivalent to much more than the amino nitrogen of arginine if it should all be liberated and is almost equivalent to all the α-amino nitrogen from the three basic amino acids of albumin as determined by the best modern methods (Calvery (13) and Vickery and Shore (22)).   The action of protaminase following pepsin hydrolysis was slightly greater than following pancreatic proteinase.   It will be interesting to investigate further the products of hydrolysis produced by this enzyme and to determine definitely whether or not free histidine and lysine as well as arginine are among them.

The hydrolysis by aminopolypeptidase and carboxypolypeptidase was extensive following proteinase or proteinase and protaminase.   With both enzymes the average amino nitrogen liberated is 30 per cent of the total nitrogen following proteinase plus protaminase.   The amino nitrogen is then equivalent to 60 per cent of the total nitrogen, while when pepsin and one of the above enzymes are used the total amino nitrogen liberated is equivalent only to 50 per cent of the total nitrogen.

The action of erepsin (dipeptidases) following the combinations mentioned above was sufficient to liberate in every case, whether determined by the Van Slyke method or by titration, enough amino nitrogen so that the average amount which could be determined after complete enzymatic hydrolysis of egg albumin was approximately 72 per cent, which with the 3.4 per cent of amino nitrogen present before hydrolysis gave a total average value of about 75 per cent.

The most accurate determinations of the molecular weight of egg albumin in various laboratories give a value of 34,000.   If the average molecular weight of the amino acids occurring in egg albumin is calculated and the weight of 1 molecule of water subtracted (since that weight is lost by the linking together of the amino acids by peptide linkage in the protein molecule), we get approximately 126.   When 34,020 is divided by 126 we obtain the value 270 which should represent approximately the number of

peptide linkages in 1 molecule of egg albumin.   When this value is used and the estimated number of peptide linkages split by each enzyme is calculated from the per cent amino nitrogen liberated, we obtain a series of whole numbers that is surprising in proportionality.   In the case of pepsin and its hydrolysis products it appears that the chief products produced by pepsin are tripeptides.   Such a concept is in direct contrast to older views but the author feels it is very probably true and will make further investigations to see if such a theory can be supported by direct evidence.

This work was carried out in the laboratory of Professor Ernst Waldschmidt-Leitz in the Institut für Biochemie, Deutsche Technische Hochschule, Prague, and the author wishes to take this opportunity to thank Professor Waldschmidt-Leitz and Dr. Schäffner for their kind advice and generous help which they gave so freely during the course of this investigation.

SUMMARY

The hydrolysis of crystalline egg albumin by means of pure proteinase of pancreas has been studied.   The extent of the hydrolysis was determined and it was found that one-third of the calculated number of peptide linkages was split.   It was further found that the number of amino groups and the number of carboxyl groups produced were the same.   Subsequent treatment with pepsin produced no further hydrolysis, while a preparation of papain-hydrocyanic acid hydrolyzed another one-third of the calculated number of peptide linkages present in the albumin.

Protaminase following proteinase liberated 6 per cent of the total nitrogen as free amino nitrogen.   Aminopolypeptidase and carboxypolypeptidase carried the hydrolysis to the same end-point following proteinase action or following proteinase plus protaminase.   The amount of amino nitrogen liberated following their action was 60 per cent of the total nitrogen.   Dipeptidase further liberated 10 to 12 per cent so that after complete hydrolysis by means of enzymes 75 per cent of the total nitrogen was present as amino nitrogen, 72 per cent of which had been liberated by enzymes.

The action of pepsin and of papain-hydrocyanic acid on crystalline egg albumin has also been studied as well as the sub-

sequent action of some other enzymes on the hydrolysis products produced by these two.

Pepsin splits the albumin molecule to such an extent that it liberates 24 to 26 per cent of the total nitrogen as amino nitrogen as estimated by the Van Slyke method or by the titration of the free carboxyl groups in 90 per cent alcohol. The rate and extent of hydrolysis is the same when coagulated albumin is used as when native albumin is used.

When protaminase is allowed to act on the hydrolysis products by pepsin a further 7 per cent of the total nitrogen is liberated as amino nitrogen. When either of the polypeptidases is used it liberates from the hydrolysis products produced by pepsin a further 24 to 25 per cent of the total nitrogen as amino nitrogen and following their action the dipeptidase present in erepsin produces a still further 24 to 25 per cent of free amino nitrogen so that the sum of the action of pepsin plus either of the polypeptidases and the dipeptidases of erepsin is the liberation of 72 to 75 per cent of the total nitrogen of egg albumin as free amino nitrogen. This value is practically the same as that obtained when either pancreatic proteinase or papain-hydrocyanic acid is used instead of pepsin.

The action of papain-hydrocyanic acid is twice as extensive as that of either pancreatic proteinase or pepsin; and following its action there is no further action by either of these enzymes.

A discussion concerning the possible nature of the products liberated by the different enzymes and the possible number of peptide linkages hydrolyzed is presented.

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IODINE AND GOITER WITH ESPECIAL REFERENCE TO THE FAR EAST

By J. F. McCLENDON

With the Assistance of Takeo Imai

The Japanese (1) are extraordinarily free from goiter, apparently due to the high iodine content of their diet of which seaweed (2) forms a constant ingredient. Since seaweed contains at least 1000 times as much iodine as any other article of food, I spent a year collecting and analyzing about 80 species of seaweed from about fourteen localities on the coast. I collected seaweed from water of the Mutsu Bay region, particularly near the Marine Biological Station of Tohoku Imperial University at Asamushi, which is near the entrance of Aomori Bay into the larger Mutsu Bay. Since this body of water has a relatively narrow entrance into Tsugaru Strait, it seems possible that seaweed might remove so much iodine from the water as to reduce its concentration appreciably, and therefore it was desirable to obtain seaweed from other localities on the coast. Groups of students very kindly collected seaweed for me, during June, July, and August, over a wide area (see map, Fig. 1).

I have numbered these localities as follows, beginning with the most southern and continuing northward: (1) Seto Marine Biological Station of Kyoto Imperial University, (2) Misaki Marine Biological Station of the Tokyo Imperial University, (3) the Mutsu Bay region, chiefly Aomori Bay near the Asamushi Marine Biological Station of Tohoku Imperial University, (4) Okudairake on the northern tip of Honshu on Tsugaru Strait, (5) Shirigishi-Uchimura-Furutakei in Hokkaido in the region of Sapporo, (6) Oshoro in the same region, (7) Otaru in the same region, (8) Kaishomai near the northern tip of Hokkaido, (9) Riyakotan just north

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of the latter, (10) Hokkaido, but of unknown locality, (11) Toshir-
ari near the center of the northwest coast of Kunashiri, the most
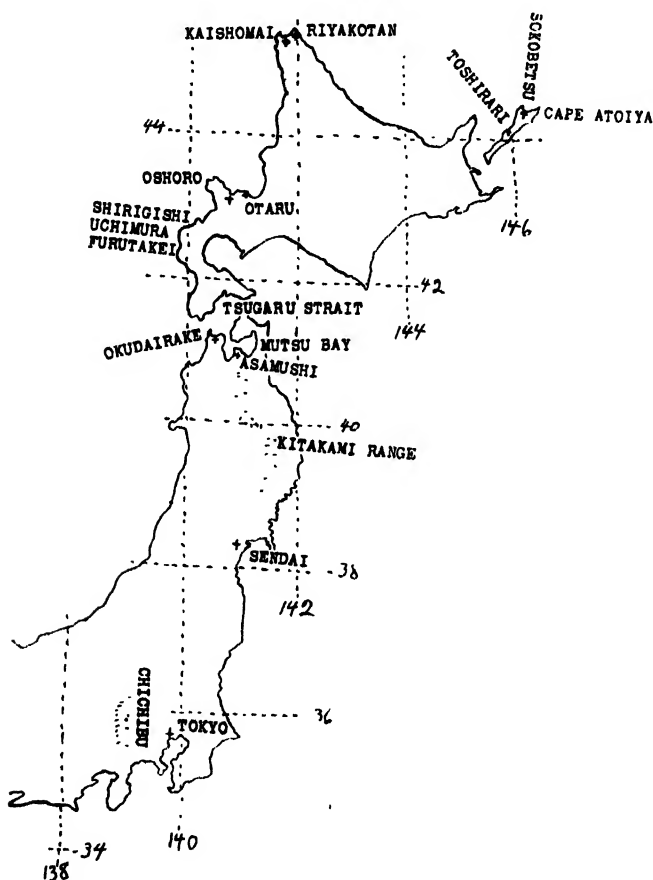southern of the Kuril Islands which extend from Hokkaido to

Fig. 1. Map of part of Japan with the names of the localities on the coast
at which seaweed was collected, and the locations of the Kitakami and
Chichibu mountain ranges, where there are a few people who do not eat
seaweed and have goiter. The figures on the map indicate the latitude and
longitude.

Kamchatka, (12) Sokobetsu near the northern tip of Kunashiri,
(13) Cape Atoiya, the northeastern tip of Kunashiri, (14) Alaid,
the most northern island of the Kuril group. The Japanese eat

nearly all of these seaweeds and what they do not eat they make into various products, such as glue for mixing with mud to plaster their houses. The iodine of most of them gets finally, either directly to the people or finds its way to the soil where it is taken up by the crops.

Owing to the very high content of iodine we do not record it in the usual way, but in mg. per kilo (parts per million). For comparison with other papers on iodine in foods three zeros should be added to these figures to bring them to micrograms per kilo (parts per billion). All the figures are on a dry basis.

In one instance it was possible for me to determine the family of an unknown seaweed by the iodine analysis. Although this is not generally true, the brown algæ contained the largest quantity and the Laminariaceæ the highest of the brown algæ and the genus *Laminaria* the highest of the Laminariaceæ. Thus the green algæ contained from 7 to 1620 mg. per kilo (Roman (3) gives 0 to 590 mg. per kilo), the red algæ from 10 to 1130 mg. per kilo (Roman (3), 0 to 1610), and the brown algæ from 17 to 7620 mg. per kilo (Roman (3), 0 to 90,000). Of the green algæ, *Codium intricatum* had much more iodine than any of the other species of the family (four genera) studied. Of the red algæ the genus *Gelidium* used for making agar (kanten) had a medium quantity of iodine (73 to 340 mg. per kilo). The genus *Grateloupia* used in preparing the most delectable foodstuff, nori, also had a medium amount of iodine, 20 to 330 mg. per kilo.

Perhaps the locality made some difference but there were such differences due to difference in age of the seaweed and individual variations that any local differences are obscured. The whole plant was analyzed since the different parts contained different amounts of iodine, except in a few cases part of the root was not obtained and a slight error arose from not obtaining quite the whole plant. Thus, individual specimens of *Laminaria ochotensis* from the same locality showed a variation in iodine from 730 to 4540 mg. per kilo, which includes all the local variations which we observed. We collected many specimens of *Undaria pinnatifida* from the Mutsu Bay region near Asamushi and found iodine from 20 to 44 mg. per kilo. The same species collected from Tsugaru Strait had a higher iodine content—from 50 to 70 mg. per kilo—but these specimens were taken from deeper water and all of the root was not

obtained.   Gautier (4) claims that seaweed may remove all of the inorganic iodine from surface water, and the specimens from Asamushi were taken near the surface.

Sargassum tortile contained a lower quantity of iodine, 14 to 23 mg. per kilo, at Seto than at Misaki, 41 to 47 mg. per kilo, but these specimens were not in perfect condition.   They were collected toward the end of the season when some of the thalli had been macerated and, perhaps, some of the iodine lost.

Certainly the species is more important than the locality in determining the iodine content, which is very well illustrated by the fact that kombu is by far the highest food in the world in iodine content.   *Laminaria ochotensis* (rishirikombu) contains 730 to 4540 and *Laminaria religiosa* contains 830 to 7620 mg. per kilo. The green alga *Enteromorpha compressa* contained only 7 and *Codium mucronatum* 6.8 to 7.3 mg. per kilo.   Also, the flowering plants (eel grass) growing in the same water with the seaweed contained very much less iodine: *Zostera nana* contained 2.1 mg. per kilo and *Halophila euphlebia*, 2.8 mg. per kilo.

The exact amount of seaweed eaten by the Japanese has not been determined.   They often eat 10 gm. (dry) or more of the seaweed at a meal.   Sometimes there are numbers of different species in one preparation and several different preparations in one meal.   Seaweed is served in a very large variety of ways under a number of different names.   Dr. E. Papellier of Kobe wrote me that he had for years advocated the eating of seaweed as a means to combat goiter, and mentioned the Japanese foods: tsukemono, kombu, arama, katsumi, wakame, kanten, haba-nori, ao-nori, somen-nori, yaki-nori, and aji-tsuke-nori.   Besides these there are other Japanese names listed in parentheses in Table I.

Method

The combustion apparatus (5) (Fig. 2) consisted of a 200 cc. Pyrex balloon combustion flask placed horizontally and with a hole blown in its bottom admitting a silica tube 30 cm. long and of 1 cm. bore containing a spiral 26 gauge platinum wire 360 cm. long. Part of the spiral extended into the flask and was large enough to admit a glazed porcelain boat 4 × 6 × 50 mm. holding the 0.1 gm. of dry, powdered seaweed.   The free end of the silica tube was inserted into a Pyrex U-tube (and sealed with asbestos fibers)

TABLE I

Determination of Iodine Content of Various Foods

For the significance of the locality figures see p. 91.

Class, family, genus, species	Locality	Iodine per kilo dry matter
		mg.
Green algæ, 7–1620		
Ulvaceæ, 7–26		
Enteromorpha compressa (awo-nori)	3	7
E. intestinalis (edauchi-awo-nori)	3	26
Ulva conglobata (hohozudi-awosa)	1	20
		25
Codiaceæ, 7–1620		
Codium mucronatum (miru)	3	7
		7
C. intricatum (motsure-miru)	1	1320
		1620
C. cylindricum (naga-miru)	1	40
		50
C. mamilliosum (tama-miru)	1	160
		240
Cladophoraceæ, 14–630		
Cladophora wrightiana (chashio-gusa)	2	590
		630
Cladophora, sp.	3	14
C. glaucescens	3	20
		26
Brown algæ, 17–7620		
Encœliaceæ, 46–67		
Colpomenia sinuosa (fukuro-nori)	3	60
		67
Colpomenia, sp.	3	60
Punctaria latifolia (haba-modoki)	3	46
Chordariaceæ, 27		
Chordaria firma (ishi-modsuku)	3	27

Class, family, genus, species	Locality	Iodine per kilo dry matter
		mg.
Dictyotaceæ, 25–210		
Padina arborescens (umi-uchiwa)	1	25
		28
P. pavonia (konaumi-uchiwa)	1	29
		30
Dictyota dichotoma (amidi-gusa)	3	43
Haliseris undulata (shiwa-yahaku)	1	70
		200
Zonaria diesingiana (shima-awogi)	1	210
Laminariaceæ, 20–7620		
Chorda filum (tsuru-mo)	3	20
Laminaria ochotensis (rishirikombu)	8	1770
	8	1960
	9	730
		980
		980
		1070
		4100
		4540
	10	2080
		2100
		2270
L. longissima (naga-kombu)	13	710
		750
L. dentigera (kumade-kombu)	14	1750
		1780
L. religiosa (hosome-kombu)	6	830
		1250
	7	7050
		7620
L. sachalenensis (karafuto-tororo-kombu)	12	2230
		2350
L coriaceæ (atsuba-kombu)	10	2310
		2330

TABLE I—*Continued*

Class, family, genus, species	Locality	Iodine per kilo dry matter	Class, family, genus, species	Locality	Iodine per kilo dry matter
		mg.			*mg.*
Laminariaceæ—*Concluded*			Fucaceæ—*Concluded*		
L. japonica (ma-kombu)	10	800	S. patens (yatsumata-moku)	1	260
		900			320
		1100	S. patens (bud)		130
		1600			130
		2000	S. aquifolium		27
Laminaria, sp.	10	1300			51
		1500	S. microcanthum (toge-moku)	1	320
		2400			380
		2500	S. tortile (yore-moku)	1	140
Alaria macroptera (chi-shima-wakame)	13	410			230
		460		2	410
A. dolichorhachis	11	450			470
		540	S. thunbergii (umitorano-wo)	1	210
Undaria pinnatifida (wa-kame)	3	44			230
		44		2	660
		37			740
		36		3	220
		44	S. confusum (fuohi-suzi-moku)	3	90
		20			100
		20	S. piluriferum (mameta-wara)	1	1120
	4	70			1130
		70	Sargassum, sp.	3	23
		50			27
		50	" "	1	660
Ecklonia kurome (kurome)	1	270			660
		280	" "	1	340
Fucaceæ, 23–1470			" "	2	1110
Sargassum serratifolium	2	1150			1150
		1110			
		1470			
		1380	Red algæ, 10–1310		
		750			
		960	Helminthocladiaceæ, 11–16		
S. ringgoldianum (ohba-moku)	2	1100	Nemalion vermiculare (umizomen)	3	11
		1300			12
S. tosænse	1	200			14
		150			16

TABLE I—*Concluded*

Class, family, genus, species	Locality	Iodine per kilo dry matter	Class, family, genus, species	Locality	Iodine per kilo dry matter
		mg			mg.
Gelidaceæ, 73–340			Rhodophyllidaceæ, 32 56		
Gelidium amansii (tengusa)	1	130	*Eucheuma papulosa* (tosa-ka-nori)	1	32
		140			38
G. subcostatum (hirakusa)	3	300	*Tichocarpus crinitus* (chiohima-tsunomata)	5	55
		340			56
G. japonicum (onikusa)	1	190	Ceraminaceæ, 1260–1310		
		240	*Ptilota pectinata* (kushi-benihiba)	5	1260
Gelidium, sp.	3	73			1310
		76	Grateloupiaceæ, 10–330		
Gigartinaceæ, 21 -390			*Schizymenia dubyi*	3	12
Chondrus ocellatus (tsunomata)	3	25			10
		21	*Grateloupia affinis* (matsunori)	3	35
Chondrus, sp.	3	36			39
		44	*G. filicina* (mukade-nori)	3	20
" "	5	59			22
		59	*G. divaricata* (kata-nori)	3	65
" "	1	31			85
		38	*G. ligulata*	3	66
Gymnogongrus furcellatus var. *japonica* (harigane)	3	150			68
Iridæa laminarioides (ginanso)	5	330	*Grateloupia*, sp	1	310
		390			330
Sphærococcaceæ, 800-900			Rhizophyllisaceæ, 100 110		
Hypnea, sp.	1	800	*Chondrococcus hornemanni* (hosobanaminohana)	1	100
		900			110
Rhodymeniaceæ, 12–18			Corallinaceæ, 29 93		
Chylocladia wrightii (o-taoyagiso)	3	12	*Amphiroa*, sp.	3	85
		12			93
C. champia parvula (watsunagi-so)	1	16	" "	1	49
		18			59
Rhomelaceæ, 114 810			*Cheilosporum*, sp.	1	67
Laurencia, sp.	3	123	*Corallina*, sp.	3	37
		114	" "	1	29
" "	1	790			32
		810	Phanerogamæ, 2–6		
Rhodomela, "	5	200	*Zostera marina* (amamo)	1	5
		210		3	6
Odonthalia corymbifera (hakesaki-nokogiri-hiba)	5	400	*Z. nana* (koamamo)	1	2
		460	*Halophila euphlebia* (oh-umihirumo)	1	3

which was bent down into a beaker of cold water and then up and connected with an absorption apparatus in which the white smoke from burning seaweed was filtered through an alundum capsule immersed in dilute $NaHSO_3$ solution.   A suction pump was used to draw the vapors through the apparatus.   If the reduced pressure was applied to the outside of the alundum, it sometimes burst, so it was applied to the inside.   The foam in the $NaHSO_3$ solution inside the alundum capsule passed up into a 200 cc. Pyrex balloon flask and broke.   A stream of oxygen from a tank was played on the seaweed by means of a Pyrex tube which passed through a mica plate which partly closed the opening of the combustion flask.
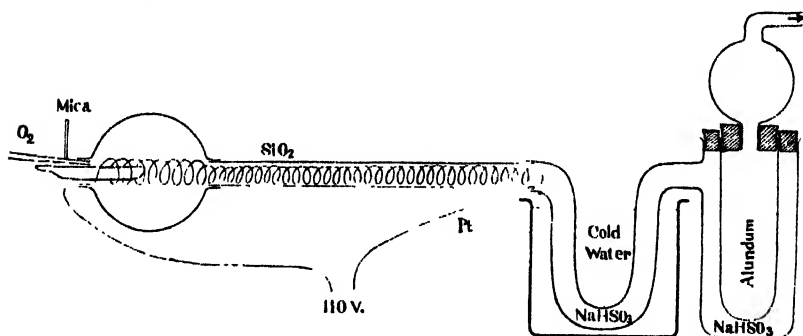
Fig. 2. Combustion tube for burning seaweed for iodine analysis

Before starting the analysis 1 gm. of $NaHSO_3$ was placed in the U-tube and enough distilled water to fill it and suction applied, thus drawing most of the solution into the alundum capsule.   The boat was placed so that only its front end was in the platinum coil. Oxygen was supplied through a needle-valve at a rate slightly less than the rate of suction at the other end.   The spiral was heated to a bright red heat by means of a 110 volt electric current and soon the seaweed ignited.   As it burned to a light colored ash, the boat was advanced until all was burned.   The ash and washings of the apparatus were evaporated to less than 10 cc. and 1 mg. of sodium azide added to decompose any nitrite that might be present.   The solution was acidified with syrupy phosphoric acid (brom-phenol blue paper being used) and boiled until the odor of hydrazoic acid and SO_2 disappeared.   It was then transferred to a 12 cc. separa-

tory funnel with minimum quantities of water, 1 cc. of pure CCl_4 and 1 mg. of $NaNO_2$ added, and shaken 200 times during 2 minutes. The CCl_4 was drawn off into a 1 cc. glass-stoppered centrifuge tube and centrifuged until clear, then placed in the left cup of a Bausch and Lomb microcolorimeter and set at 20 mm. A standard containing 0.1 mg. of iodine in 1 cc. of pure CCl_4 was placed in the right cup. Ten readings were made and added.

The separatory funnel was inverted and the drop of watery solution in the hole in the stop-cock allowed to run back, then placed upright, and 1 cc. of CCl_4 added and a second extraction made and analyzed as was the first. The total iodine (6) $= x_1^2/(x_1 - x_2)$ where x_1 is the sum of ten readings on the first extract and x_2 the sum of ten readings on the second extract. If the result is divided by 2000 it gives the percentage of iodine in the dry seaweed.

SUMMARY

In a study of goiter around the world, Japan was the only nongoitrous country that was found, there being about one goiter per million Japanese. The iodine intake of the Japanese is very high and certainly more than double that of any other peoples, as seaweed is a constant constituent of their diet and seaweed has about 1000 times as much iodine as any other food. An analysis of the iodine content of 79 species of seaweed from fourteen localities on the coast is given.

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THE RÔLE PLAYED BY BILE IN THE ABSORPTION OF VITAMIN D IN THE RAT

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The work of Pavlov (1), Looser (2), Wisner and Whipple (3), Düttmann (4), Dietrich (5), Gilbert (6), Bushbinder and Kern (7), Seidel (8), and Rigano (9) points to the fact that exclusion of bile from the intestinal tract of man and of certain other animals leads to osteoporosis. Two explanations to account for this phenomenon have been advanced. Klinke (10) postulated that it is due to faulty calcium absorption resulting from the formation of calcium soaps. Seifert (11) has suggested that in the absence of bile vitamin D is not absorbed which in turn leads to a negative calcium and phosphorus balance. Support to the latter hypothesis was furnished by Tammann (12) who showed that parenteral administration of vitamin D alleviated the condition.

There is a good deal of evidence which points to the rôle played by bile in the absorption of the lipids. Little or no fat is absorbed when bile is totally excluded from the intestinal tract (13). The absorption of cholesterol is markedly decreased in icteric patients (14). The work of Schönheimer and others (15) indicates that cholesterol absorption is facilitated by desoxycholic acid. Curiously, however, plant sterols are not absorbed in appreciable amounts from the alimentary tracts of some animals (16). When these compounds are fed to the rabbit together with cholesterol, only the latter substance is found in the chyle from the thoracic duct. Non-irradiated ergosterol is apparently not absorbed or at least not stored in the rat, mouse, and rabbit (17). It is absorbed in small amounts by the hen (18). On the other hand, irradiated ergosterol or vitamin D must be absorbed since it is administered

therapeutically by mouth.[1]   From the close relationship between the fat-soluble vitamins and the sterols it might be expected that the absorption of the former would also depend upon the carrier action of bile.

Several papers dealing with this subject have been published from this laboratory.   In the case of vitamin A it was shown that under the conditions of the experiments in which bile was withheld from the intestinal tract by ligating and sectioning the common bile duct, sufficient vitamin A was absorbed to restore the vaginal smear picture of rats which had been depleted of this factor to normal (19).   These experiments are perhaps subject to criticism from the standpoint that traces of bile may have been present in the intestinal tracts of the animals for several days following the operation.   Experiments which were carried out on two chole-cystonephrostomized dogs showed that the negative calcium and phosphorus balances could be changed to positive ones by the subcutaneous administration of viosterol, indicating that in the absence of bile from the intestinal tract, little or no absorption of this factor takes place (20).   These experiments have now been extended to rats.

A search of the literature failed to reveal any reference to bile fistula rats.   The rat does not possess a gallbladder; thus a chole-cystonephrostomy is not possible.   External fistulas are trouble-some inasmuch as constant care must be taken to avoid infection and to prevent access of the animal to the bile.   In our experience, such animals usually die within 3 to 4 days following the operation. We, therefore, turned our attention to the development of an internal fistula.

The absorption which takes place from the lower colon is small and consists primarily of water and a small amount of sugars and possibly also amino acids.   There is no evidence of fat absorption in the lower colon.   Thus, if the bile duct could be connected to the lower colon, the greater part of the intestinal tract could be freed from bile.   After some practise, it was found possible to do this in the following manner.

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[1] In this connection, see Koehne, M., and Mendel, L. B., *J. Nutrition*, **1,** 399 (1928–29).

Adult rats were fasted for 24 hours without food or water. Ether was used as an anesthetic. A medial incision was made, and the common bile duct was cut from the duodenum in such a manner as to take a small piece of the duodenum with it. The bile duct was passed under the duodenum and sewed into a small hole made in the descending colon. The opening in the duodenum, as well as the incision, was then surgically closed. Food and water were withheld for another 24 hours. Great care was necessary in order to avoid biliary obstruction. Only animals free from jaundice, as shown by absence of bile pigments in the urine and skin 48 hours after the operation, were used in this work. Of 63 animals which were operated upon, only twenty-one of them met the above conditions. The remaining forty-two animals showed extended periods of jaundice and were discarded. Fifteen of the twenty-one successfully operated animals lived for a sufficient length of time to obtain balances of four or more periods. For the sake of space economy, data obtained on three animals only are presented in this paper. They are illustrative of the general trend of the experimental results. All animals had been on the following diet for periods ranging from 4 to 9 weeks previous to the beginning of these experiments: wheat (ground fine), 30 pounds; fish-meal, 5 pounds; milk powder, 5 pounds; alfalfa meal, 1 pound; sodium chloride, 100 gm.; calcium carbonate, 100 gm.

This diet was selected because experience had shown that the bile fistula rat can tolerate it well. Its fat content is low by necessity. All dietary essentials are included. The diet was mixed and remixed until repeated analysis showed it to have a constant calcium and phosphorus content of 0.993 and 0.623 per cent respectively. It has a calcium to phosphorus ratio of 1.6.

All animals were kept in standard individual metabolism cages in an air bath maintained at 26°. An electric fan was used to keep the air in motion. All air was drawn into the air bath through a cotton filter and forced out of doors through a window.

All mineral balances, except four, were carried out over a period of 1 week. The food consumption was determined daily. At the end of each period, the wire screens were removed from the cages and boiled with water to remove the last traces of excreta. The washings were evaporated to dryness and added to the week's

sample. They were ashed at a dull red heat, taken up in dilute HCl, and calcium and phosphorus determinations were made on aliquot portions by standard methods (21, 22).

Two 1 week balance periods were carried out on a number of animals prior to the operation to test the adequacy of the diet. The results show that all of the animals were in slightly positive calcium and phosphorus balance during this period. The diet therefore seemed adequate and these normal periods were omitted in the later experiments.

The animals were then operated upon and the balances continued for periods of 1 to 4 weeks, after which the animals received Squibb's viosterol 250 D in oil, orally or subcutaneously, with or without desoxycholic acid. The viosterol solution contained 3333 rat units per gm. of solution.

Autopsies were performed on all animals at the conclusion of the experiments to verify the operation.

Results

It is evident from the data given in Tables I and II that, although all of the rats were in slight calcium and phosphorus balances during the preoperative period, as a result of the bile fistula operation the calcium and phosphorus balances on two of the animals (Rats 708 and 709) became negative, while the third (Rat 706) showed a negative phosphorus balance but a slightly positive calcium balance. The results are essentially in accord with the negative calcium and phosphorus balances obtained with bile fistula dogs (20). The quantitative variations in the calcium and phosphorus balances obtained on different animals are probably due to the amounts of vitamin D stored by the animals at the time the experiments were begun.

Oral administration of 333 rat units of viosterol to Rat 708 (see Table I) did not lead to an appreciable shift in the negative calcium and phosphorus balance values. In fact, for two periods the animal was in negative calcium and phosphorus balance despite the oral administration of vitamin D. Results essentially in accord with the above were obtained on five other animals, the data for which are not included here. The conclusion is drawn that the absorption of irradiated ergosterol is markedly reduced in the bile fistula rat. On subcutaneous administration of vitamin D to Rat

708, the negative calcium and phosphorus balances became positive. This is likewise true of Rat 709 (see Table I) and Rat 706 (see Table II). However, on continued administration of 333 rat units subcutaneously, the calcium and phosphorus balances on Rats 708 and 709 became either negative or decreased in amounts over the previous period. This is apparently due to

TABLE I

Calcium and Phosphorus Balances on Rats 708 and 709

Treatment	Body weight	Experimental period	Food intake	Ca intake	Ca excreted	Ca balance	P intake	P excreted	P balance	Ca P ratio
	gm	*days*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm*	*gm*	*gm.*
Rat 708										
Fore period.	290	1– 7	89 1							
None.	289	8– 14	90 1	0 89	0 88	+0 01	0 47	0 45	+0 02	0 50
"	294	15– 21	95 6	0 99	0 93	+0 06	0 50	0 47	+0 03	1 66
Internal bile fistula made 22nd day	281	22– 28	73 3	0 73	0 73	0 00	0 38	0 38	0 00	
None	257	29– 35	99 9	0 99	1 03	–0 04	0 62	0 64	–0 02	2 00
333 rat units viosterol orally daily	247	36– 42	130 3	1 29	1 25	+0 04	0 82	0 77	+0 05	0 80
" "	239	43 49	107 1	1 06	1 12	–0 06	0 67	0 70	–0 03	2 00
" "	240	50– 56	90 1	0 90	0 93	–0 03	0 56	0 61	–0 05	0 60
333 rat units viosterol subcutaneously 3 times per wk.	259	57– 63	143 4	1 42	1 04	+0 38	0 89	0 63	+0 26	1.50
" "	248	64– 70	133.5	1 33	1 75	–0.42	0 83	1 09	–0 26	1 60
None	248	71– 77	113 4	1 13	1 14	–0 01	0 71	0 71	0 00	
"	249	78– 84	117 1	1.16	1 16	0 00	0 73	0 74	–0 01	
1000 rat units viosterol subcutaneously 85th day	259	85– 91	149 1	1 48	1 38	+0 10	0 93	0 83	+0 10	1 00
None	245	92– 98	160.5	1 59	1 58	+0 01	1 00	0 97	+0 03	0 30
"	241	99–105	126 3	1 25	1 30	–0 05	0.79	0 84	–0 05	1 00
95.5 mg. desoxycholic acid + 3180 rat units viosterol in food.	240	106–112	95.5	0 95	0 87	+0 08	0 60	0 55	+0 05	1 60
None	236	113–119	113 6	1.13	1 19	–0 06	0 71	0 80	–0 09	0 66
"	261	120–126	141.2	1 40	1 45	–0 05	0.88	0 92	–0 04	1.25

TABLE I—*Concluded*

Treatment	Body weight	Experimental period	Food intake	Ca intake	Ca excreted	Ca balance	P intake	P excreted	P balance	Ca·P ratio
	gm	*days*	*gm.*	*gm.*	*gm.*	*gm*	*gm.*	*gm.*	*gm.*	
					Rat 709					
Fore period	260	1– 7	99 9							
None.	262	8–14	101 0	1 00	0 97	+0 03	0.53	0.52	+0 01	3 00
"	261	15–21	112 0	1 11	0 95	+0 16	0.58	0 53	+0 05	3 20
Internal bile fistula made 22nd day. .	248	22–28	146 1	1 45	1.40	+0 05	0 76	0.70	+0 06	0 84
None	238	29–35	168 8	1 68	1.71	–0 03	1 54	1 62	–0 08	0 38
333 rat units viosterol subcutaneously daily	232	36–42	187 8	1 85	1 34	+0 51	1 17	0 80	+0 37	1 40
" "	200	43–49	105.3	1 05	1 09	–0 04	0 74	0 66	+0 08	
" "	158	50–56	15 6	0 16	0 34	–0 18	0 10	0 27	–0 17	1 05

Rat 709 died 56th day. Autopsy; liver darkened on under side and edges; bile duct enlarged but operation was successful.

the high dosage of viosterol employed. When smaller amounts such as a single dose of 1000 units of irradiated ergosterol during the period of a week were administered subcutaneously, the negative phase was decreased somewhat (Rat 706) or entirely absent as in Rat 701, data for which have not been included in this paper.

Rats 708 and 707 (data for the latter animal have not been included) offer an interesting comparative study. Both animals were treated similarly throughout. Rat 707 became jaundiced just after the first treatment period and continued so to its death. Both animals reacted similarly to all treatments except that the negative phase following the subcutaneous treatment in Rat 707 was much drawn out. Rat 708 returned to balance the week after the subcutaneous administration of vitamin D was discontinued, while Rat 707 continued in negative calcium and phosphorus balance during the 2 remaining weeks of its life. In the case of the latter animal, the elimination of bile was much less than in the case of the rat with a bile fistula. Retention of vitamin D probably occurred for a more extended period of time in the jaundiced rat.

The evidence in the introduction, as well as in the preceding discussion, points to the rôle of desoxycholic acid as a probable carrier of vitamin D across the intestinal wall.   A series of experiments was carried out to determine this more directly.   Purified

TABLE II

Calcium and Phosphorus Balances on Rat 706

Treatment	Body weight	Experimental period	Food intake	Ca intake	Ca excreted	Ca balance	P intake	P excreted	P balance	Ca P ratio
	gm	days	gm.	gm.	gm.	gm	gm	gm	gm.	
Fore period	290	1- 7	90 1							
None	290	8-14	84 3	0 84	0 78	+0 06	0 44	0 43	+0 01	6 00
"	288	15-21	85 8	0 85	0 89	-0 04	0 45	0 38	+0 07	0 60
Internal bile fistula made 22nd day	236	22-28	38 2	0 38	0 41	-0 03	0 24	0 29	-0 05	0 60
None	224	29 35	37 2	0 37	0 37	0 00	0 23	0 26	-0 03	
" .	227	36-42	81 6	0 81	0 77	+0 04	0 51	0 52	-0 01	
333 rat units viosterol subcutaneously daily	220	43-49	89 3	0 89	0 89	0 00	0 56	0 58	-0 02	
" "	224	50-56	112 8	1 12	0 99	+0 13	0 70	0 65	+0 05	2 60
" "	238	57-63	115 6	1 15	1 10	+0 05	0 72	0 69	+0 04	1.25
None .	241	64-70	120 0	1 19	1 28	-0 09	0 69	0 79	-0.10	0.90
" .	247	71 -77	135 2	1 34	1 41	-0 07	0 84	0 86	-0 02	3 50
1000 rat units viosterol subcutaneously 78th day	256	78-84	112 7	1 12	1 24	-0 12	0.70	0 76	-0 06	2 00
None . ..	256	85-91	112 4	1 12	1 30	-0 18	0.70	0 76	-0 06	3 00
" . . .	259	92-98	117 7	1 17	1 23	-0 06	0.73	0 76	-0 03	2 00
1666 rat units viosterol subcutaneously 99th day	261	99-105	117.5	1 17	1 22	-0 05	0 73	0 74	-0 01	5 00
None	265	106-112	126 3	1 25	1 28	-0 03	0 79	0 87	-0 08	0 40

Animal killed 118th day.

desoxycholic acid and viosterol were mixed with the diet so that each gm. of diet contained 1 mg. of desoxycholic acid and 33.3 rat units of viosterol.   It will be noted that as a result of this treatment the negative calcium and phosphorus balances of Rat 708 became positive.   Similar results were obtained with Rat 701,

data for which have not been included here. In a number of preliminary experiments the dosage of desoxycholic acid which was administered was not only smaller than in the above experiments but it was administered in a single daily dose by stomach tube. In the latter experiments the calcium and phosphorus balances continued negative. It is of course difficult to estimate the amount of bile acids which are effective in the normal animal since the bile acids circulate and a small amount can perform the same function as larger doses when administered to a bile fistula animal. In the latter case, not only is the possibility of recirculation excluded, but a loss of bile acids occurs as well.

The bile fistula animal appears to require a larger dosage of viosterol to maintain itself in positive calcium and phosphorus balance than does the normal animal. This increased demand was evident in our bile fistula dogs (20). During the preoperation periods, the diet used in our experiments with bile fistula rats was adequate, yet, soon after the operation, the animals tend to go into negative calcium and phosphorus balance, again showing an increased demand for vitamin D.

The bile fistula rats which were treated with 1000 to 2330 rat units of viosterol per week for periods of 2 to 3 weeks, invariably lost considerable quantities of calcium and phosphorus during the latter part of the treatment period (see Rats 706 and 709, Tables I and II). In order to test out the effect of a similar dosage on the normal rat, the following experiments were performed.

A group of six normal rats was placed on Steenbock's (23) rachitic diet (Ration 2965) for a period of 6 weeks to reduce their vitamin D stores. They were then placed in standard metabolism cages and complete calcium and phosphorus balances were carried out under conditions similar to those of the bile fistula rats. A modified Steenbock's rachitic diet was used containing 1.013 per cent of calcium and 0.273 per cent of phosphorus. This diet has a calcium to phosphorus ratio of 3.71. The toxic effects of viosterol overdosage are less on a diet of optimal calcium, low phosphorus content than they are on an optimal calcium, optimal phosphorus-containing diet (24). Any toxic effects on the viosterol-treated animals on a diet with a calcium to phosphorus ratio of 3.71 would have been greater with a ratio of 1.6. We are therefore justified in comparing this series of experiments with those of the bile

fistula animals even though the calcium to phosphorus ratios of the diet are different.   The actual differences in viosterol tolerance are probably greater than this comparison indicates.

The results of the experiments are given in Table III.   During the 2 weeks of the control period most of the animals showed small negative balance values.   Two animals (Rats 801 and 805) received 1666 units of viosterol subcutaneously at the beginning of the third weekly period.   Both showed positive balance values

TABLE III

Calcium and Phosphorus Balances on Normal Adult Rats on Steenbock Rachitic Ration 2965, and Effect Thereon of Administration of Viosterol Subcutaneously

Rat No	Con-stituent analyzed for	Control period		Viosterol ad-ministered 3rd wk	Results 3rd wk	Viosterol ad-ministered 4th wk	Results 4th wk	Results 5th wk.*
		1st wk	2nd wk					
		gm.	*gm.*	*rat units*	*gm*	*rat units*	*gm.*	*gm*
801	Ca	+0 08	−0 03	1666	+0 04	None	−0 03	−0 02
	P	0 00	−0 02		+0.02		−0 03	−0 03
802	Ca	−0 02	0 00	333 daily	−0 07	"	−0 05	−0 06
	P	−0 03	+0 01		−0 20		−0 17	−0 05
803	Ca	0 00	+0 01	333 daily	−0 13	"	+0 01	−0 03
	P	−0 01	−0 01		−0 15		−0 11	−0 02
804	Ca	−0 10	−0 03	333 daily	−0 01	333 daily	−0 14	−0 12
	P	−0 03	−0 02		−0 10		−0 16	−0 10
805	Ca	+0 06	−0 06	1666	+0 12	None	−0 08	−0 04
	P	+0 05	−0 03		+0 03		−0 13	−0 03
806	Ca	−0 02	0 00	333 daily	−0 13	333 daily	−0 02	−0 03
	P	−0 01	−0 02		−0.12		−0 10	−0 04

* No treatment was given during the 5th week.

for the week.   During the 4th week the balances again became negative.

Rats 802, 803, 804, and 806 received a daily dosage of 333 rat units of viosterol subcutaneously during the 3rd week.   All showed negative balance values during this period.   In the case of Rats 802 and 803, the dosage was discontinued during the 4th week with the result that the negative balance values decreased.   In Rats 804 and 806, the viosterol dosage was continued through the 4th week period.   Rat 804 showed an increased loss of both calcium and phosphorus, while Rat 806 showed a decreased loss.

It is thus seen that positive shifts in the balance values were obtained only when a single dose of 1666 rat units of viosterol during the week was administered.   When the larger dosages (2332 units) were administered, a marked loss of both calcium and phosphorus invariably followed.

In the case of the bile fistula rats, positive balances values were obtained when 333 rat units of viosterol were administered subcutaneously three times per week for the 1st week to Rat 708, and when 333 rat units were administered daily to Rat 709 during the similar period.   These data, while not directly comparable, give additional weight to the hypothesis that the bile fistula animal requires more vitamin D than does the normal animal.   The decided negative phase of icteric Rat 707 during the 2nd week when 333 units of viosterol per week were administered subcutaneously (data not given here) points strongly to the fact that the smaller response to a like dose of viosterol is due to increased excretion of the vitamin by the bile fistula rat.   The most probable channel is through the bile.

It is recognized that in experiments of the type recorded in this paper not only considerable errors in the estimation of small amounts of calcium and phosphorus enter but individual variations in the response of different animals to the same dose of viosterol as well as possible differences in the flow of bile must be considered. The conclusions are based on general trends rather than on precise quantities.   It appears, however, that the data which were obtained in these experiments point to the fact that the bile acts as a carrier of vitamin D across the intestinal wall of the rat and that in the absence of bile from the small intestines as in the case of the bile fistula rat little or no vitamin D is absorbed.   Absence of bile from the intestinal tract is followed by a loss of calcium and of phosphorus.

The complete data, of which only a part has been included in this paper, are on file in the University of California library.

SUMMARY

The results of a study of the absorption of vitamin D in choledochocolostomized rats when calcium and phosphorus balances are used as the criterion for absorption are reported.   The following are indicated:

1. Bile fistula rats tend to be in negative calcium and phosphorus balance soon after the operation.

2. Little or no irradiated ergosterol is absorbed from the intestinal tract of the bile fistula rat.

3. Desoxycholic acid when administered by mouth can serve as a carrier of irradiated ergosterol across the intestinal wall of the bile fistula rat.

4. In the presence of bile, the absorption of irradiated ergosterol which takes place from the descending colon of the rat, as shown in these experiments on rats with bile fistulas, is small or absent.

5. The hypothesis is advanced that the increased need for antirachitic factor by the bile fistula animal is due to increased excretion of vitamin D which probably proceeds by way of the bile.

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GASTRIC JUICE

I. STUDIES ON THE PROTEINS OF THE GASTRIC JUICE OF HUMANS

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INTRODUCTION

The studies to be reported were undertaken with the idea of throwing more light on the proteins of the gastric juice. It has been found that they can be precipitated by tungstic acid at pH 3.5 and that, although heating coagulated a protein, there remained in solution a heat-uncoagulable substance which gave a positive biuret reaction. Interest in the subject was stimulated by the investigations on the non-protein nitrogen constituents of the gastric juice (1).

As a result of the investigations to be reported, it has been found that there are present in the normal human gastric juice, in addition to the proteins of the mucus, two protein-like bodies.

The first has been called gastroglobulin and is precipitated by magnesium sulfate at one-half saturation (further saturation does not increase the yield of protein) by an acetic acid and acetone mixture, and by heat. It does not contain a carbohydrate. Although in its chemical analysis it resembles a mucoprotein, it appears to be a distinct substance. It has been crystallized from various media.

The second protein-like material is precipitated from the acetic acid-acetone filtrate and from the heated gastric juice, after globulin has been removed, by tungstic acid at pH 3.5. When this second precipitate is hydrolyzed in 2 per cent H_2SO_4, a compound is liberated which exhibits typical carbohydrate properties; namely, it reduces alkaline copper sulfate and forms osazone-like

bodies when treated with phenylhydrazine.   A small amount of gas was occasionally seen after fermentation with yeast.   Furthermore, this protein-like precipitate contains all of the carbohydrate which may be freed by hydrolysis, and further hydrolysis of the tungstic acid filtrate yields no such reducing body.   There is no direct evidence to support the idea that one is dealing here with a single chemical compound since this material was not isolated in pure form.

In addition to these observations, it has been noted that there is present in the normal human gastric juice a protein-like body which is precipitated from the filtered gastric juice at pH 6.8 to 7.0. After hydrolysis, a carbohydrate body is liberated.

<div align="center">EXPERIMENTAL</div>

<div align="center">*Methods*</div>

The gastric juice used in these determinations was collected by duodenal tubes which were placed in the dependent portion of the stomach of individuals who had been fasting for at least 15 hours. The material was collected by siphonage and suction into narrow cylinders that were packed in ice.   Immediately after cessation of flow, the extracted specimen was filtered in the ice box.   Only pooled specimens containing normal amounts of free HCl were used in the work reported here.   All specimens containing any bile contamination were rigorously discarded.   All saliva was collected and patients were cautioned not to swallow any.

Nitrogen was determined by Nesslerization after the micro-Kjeldahl method of Folin and Farmer.   This was modified to the extent that the ammonia was distilled.   The standardization of this method has been reported (2).   Proteins were removed by the method reported (1) and non-protein nitrogen was determined upon the filtrate by the method of Folin and Wu (3).   Hydrogen ion concentration was determined according to Clark and Lubs (4).   The commonly accepted methods for the biuret, Millon, Hopkins-Cole, and xanthoproteic reactions were used.   Sugar was estimated by the method of Folin and Wu (3) and expressed in terms of glucose.   Osazones were produced by the phenylhydrazine reaction.

Filters were prepared by immersing a Berkefeld candle in a 5 per cent solution of pyroxylin in glacial acetic acid.   Suction was

exerted for 1 minute. The coated candle was allowed to drain and was then plunged into cold water. After thorough hardening, air was slowly admitted into the submerged candle. The filter was thoroughly washed with water until the washings became neutral.

The material to be filtered was placed in a narrow cylinder and as the fluid was sucked through, water was added until the filtrate was free from salt and ammonia.

Celloidin bags were prepared by the formula of Pierce (5). They were allowed to dry in a vacuum for 1 hour before being placed in water. If this period was not allowed, the membrane was permeable to gastric proteins.

Precipitation of a Protein-Like Material at pH 6.8 to 7.0

The hydrogen ion concentration of the contents of a series of tubes containing pooled gastric juice was so adjusted that it ranged in small increments from that obtained in the juices as extracted, about pH 1.4 to 7.7. The volume of the series was made equal throughout. It was clearly demonstrable to the naked eye that near the neutral point a precipitate was formed. At pH 6.8 to 7.0 it was most intense and disappeared in more alkaline range. Photographs were made to demonstrate the Tyndall effect in a number of such series, all of which showed the same condition. After filtration through Whatman No. 42 paper, estimations of nitrogen were made upon the filtrate and precipitate from each tube of the series. The data are shown in Table I. It is evident that at pH 6.9 the largest amount of nitrogen was precipitated. This material gave a positive biuret reaction and, after hydrolysis in 2 per cent H_2SO_4 for 2 to 3 hours in a water bath, reduced alkaline copper sulfate.

Preparation of Gastroglobulin

By Salting—A series of tubes containing a mixture of filtered human gastric juice extractions was prepared as described above. The pH ranged from 1.4 to 7.7, and a precipitate was seen at about pH 6.9. The data shown in the first two columns of Table I were obtained from this series. After the amounts necessary for these estimations had been removed, the remaining contents were half saturated with $MgSO_4$. A heavy precipitate was found in the

tubes of low pH. This tended to disappear as the contents became more alkaline. The contents of the tubes were filtered through Whatman No. 42 paper, and the total nitrogen was determined on each portion. The predetermined non-protein nitrogen was subtracted from the filtrate. A typical experiment may be

TABLE I

Mixture XIII. Effect of Changing pH of Gastric Juice and of Half Saturating Material So Adjusted with MgSO₄

Gastric juice + NaOH				Half saturation with MgSO₄			
pH	Tyndall effect	Protein N per 100 cc. gastric juice		pH	Protein N per 100 cc. gastric juice		Remarks
		Precipitate	Filtrate		Precipitate	Filtrate	
		mg.	*mg.*		*mg.*	*mg.*	
2 0	+			3 6	15.39	5 04*	
2.4	+			4 5	14 28	6 04	
2 6	+			5 2	11.16	9.43	
3 4	+			5 4	9 99	10 36	
3.5	+			5 7	6 93	13 36	
4 0	+			6 0	5 74	15 00	
4.4	+			6 2	5 36	15 36	
5.0	+			6 5	3 85	16.30	Contents of tube
5.3	+			6 9	3 60	16.80	practically
5 5	+			7 0	3 42	16 65	clear
6.3	+			7.1	3.20	17.25	
6 5	++	1.87	19 2*	7 0	2 83	17.54	
6 7	++	2 64	18.1				No precipitate
6 9	++++	4 40	16.1				
7.0	++	3.10	17.3				
7.2	++	2.90	18 0				
7 4	+	2.60	18 4				
7 7	+	1 80	19.3				

* Non-protein nitrogen has been deducted from both filtrates.

seen in Table I. The pH values of the MgSO₄ mixtures are somewhat inaccurate owing to the heavy concentration of the salts. This fundamental inaccuracy is constant throughout the series and therefore the pH values have a direct comparative basis. At pH 3.6 the largest amount of nitrogen-containing material was precipitated. The nitrogen content of precipitates decreased as the

pH varied from 3.6. This material gave positive reactions to the biuret, xanthoproteic, Hopkins-Cole, and Millon tests. It did not reduce alkaline $CuSO_4$ after hydrolysis. It was called gastroglobulin.

By Dialysis—Studies were made after dialysis in the following manner. Fourteen tubes containing filtered, limpid gastric juice at room temperature were adjusted to various hydrogen ion concentrations ranging from pH 1.4 to 6.3, and the volumes were made equal. To each was added an equal amount of a saturated solution of recrystallized $MgSO_4$. After standing in the ice box overnight the tubes were filtered and the precipitate (gastroglobulin) was redissolved in 20 cc. of H_2O. The protein nitrogen was determined on 3 cc. of the redissolved precipitate (see Table II). The remainder was placed in separate celloidin sacs, and allowed to dialyze until free of sulfate. Each dialyzed portion was then analyzed for nitrogen. The figures are shown in Table II.

It was found that gastroglobulin was recovered almost quantitatively, and that it continued to give positive reactions with the biuret, Hopkins-Cole, xanthoproteic, and Millon tests.

By Acetic Acid and Acetone—To filtered, limpid gastric juice, sufficient glacial acetic acid was added to make a 2 per cent concentration. At times a very slight cloud was seen which was thought to be due to a small amount of mucoprotein. After thorough mixing, one-half the volume of acetone was added. This caused a flocculent precipitate to appear, which at times could be removed through centrifugation, while at other times filtration was necessary.

The precipitate was taken up in water and brought into solution by the addition of a small amount of NaOH. This usually occurred at about pH 5.6. The material gave positive responses with the biuret, Millon, Hopkins-Cole, and xanthoproteic tests. At times it reduced alkaline $CuSO_4$ after hydrolysis in 2 per cent H_2SO_4, but after one reprecipitation at pH 3.5, its $CuSO_4$-reducing ability was lost.

Properties of Gastroglobulin

To obtain sufficient gastroglobulin for the determination of its properties the following methods were repeatedly followed. (a) Large quantities of gastric juice were one-half saturated with

MgSO$_4$ at pH 3.5.   After this material had stood for several days in tall cylinders, the precipitate could be easily recovered by siphoning off the supernatant fluid and then removing the remaining fluid by filtration through Whatman No. 42 filter paper.   The gastroglobulin was suspended in water.   (b) Large amounts of gastric juice were precipitated by acetic acid and acetone as pre-
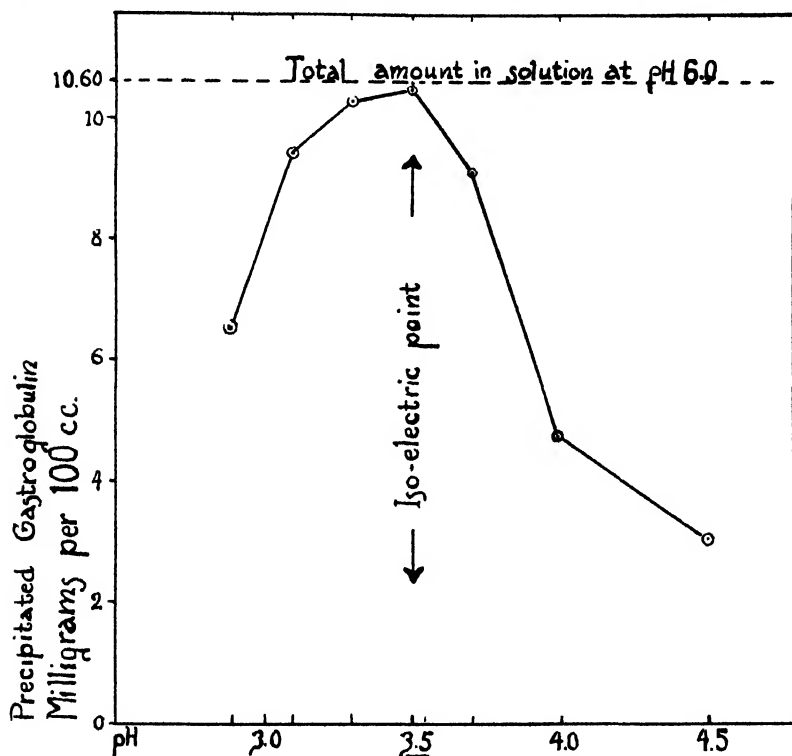
CHART 1. Effect of changing pH on the solubility of gastroglobulin.   At pH 3.5 only a minimal amount remained in solution.

viously described.   The tall cylinders containing the mixture were placed in the ice box for several days.   At the end of this time the supernatant fluid was siphoned off and used for further studies. The gastroglobulin was freed of the small remaining filtrate by filtration and suspended in water.

Both precipitates were dialyzed through thoroughly washed

Berkefeld candles which had been permeated and surrounded by an acetocelloidin membrane. Suction was continued until the dialysates were free from sulfates and chlorides. At this point estimation of nitrogen showed that the gastroglobulin was recovered almost quantitatively from the residue.

The compounds were brought completely into solution with 0.1 N NaOH at about pH 5.4 and remained so until about pH 8.5. The solutions gave positive results to the biuret, xanthoproteic, Hopkins-Cole, and Millon tests. After hydrolysis with 2 per cent H_2SO_4, they did not reduce copper sulfate nor did they produce gas on fermentation. No osazones were obtained.

They were coagulated by heat and were insoluble in water, alcohol, and ether. They were soluble in dilute acids and alkalies.

Isoelectric Point—The isoelectric point was determined upon the dissolved material by adjusting to various hydrogen ion concentrations the contents of several tubes. The results may be seen in Chart 1. The control estimation of the gastroglobulin was 10.62 mg. of N per 100 cc. The amount recovered at pH 3.5 was 10.41 mg. of N per 100 cc. This experiment was repeatedly corroborated on different mixtures of gastric juice.

Chemical Analysis—Chemical analysis of the gastroglobulin was made after two methods of precipitation and dialysis, as described above, and repeated precipitation at the isoelectric point.

Both of these protein substances were washed with alcohol and ether and then dried in a vacuum desiccator. It will be shown later (Table IV) that both methods of precipitation of gastroglobulin yield quantitatively identical amounts.

The results of analysis were as follows:

After $MgSO_4$ precipitation   C 53.14, H 6.44, ash 1.82, N 12.8, S 1.37, P 0.00
After acetic acid and acetone precipitation   " 51.83, " 7.21, " 2.35, " 11.48, " 1.76, " 0.00

Crystallization—The gastroglobulin, which had been obtained by ultrafiltration through a Berkefeld filter coated with a film of acetic acid-pyroxylin solution, was used as the starting material. It was dissolved at about pH 6. Several methods were successful in the production of crystals.

Brucine-Pyridine-Ammonia Method of Abel, Geiling, Rouiller, Bell, and Wintersteiner (6), with Modifications—(a) 20 cc. of mate-

rial containing about 25 mg. of protein nitrogen were brought to a
2 per cent concentration with acetic acid.   To this mixture were
added 40 cc. of a mixture containing 1 gm. of brucine in 18 cc. of
N/6 acetic acid.   A small precipitate was noted; 20 cc. of a 13.5
per cent solution of pyridine were then added.   The precipitate
was removed through centrifugation and 10 cc. of 0.65 per cent
ammonia were added.   The pH was adjusted to 3.5 and the flask
was placed in the ice box.   Within 24 to 48 hours a small precipi-
tate was found.   The precipitate contained crystals which were
pyramidal, hexahedral, and quite clear.   Other less well formed
crystals were found but approached this type.   They closely
approached the pepsin crystals described by Northrop (7) (see
Fig. 1).   (b) When brucine prepared as above was added in the same
proportion to the gastroglobulin suspension which had not been
previously acidified, a precipitate was found at times.   This was
especially true of the gastroglobulin which had been dialyzed
against 0.5 per cent saline, as was tried at one time.   Whether a
precipitate was found or not, pyridine was then added as above.
Any precipitate was removed through centrifugation; 20 cc. of
ammonium hydroxide, 0.65 per cent, were added to the filtrate,
and again any precipitate was removed through centrifugation.
The supernatant fluid was adjusted to pH 3.5 with glacial acetic
acid and allowed to stand at room temperature or in the ice box.
After 24 to 48 hours a precipitate was formed which was found to
contain crystals of the same shape as those described above.   After
a period of weeks more crystals were formed.

Crystallization at Isoelectric Point—It was possible to obtain
crystals by allowing the solution to remain 5 to 10 days in the ice
box at pH 3.5.   This was done by adding NaOH to the water sus-
pension of gastroglobulin to bring it into complete solution.   HCl
was then added to pH 4.5, the precipitate was removed through
centrifugation, and the supernatant fluid, after being adjusted to
pH 3.5, was placed in the ice box.   A small yield of crystals was
obtained which were different from those obtained by the brucine
method.   They were octahedral and more compact.   In many
instances they resembled in appearance the urease crystals of
Sumner (8) (see Fig. 2).

Crystallization by Alcohol—The intial precipitate obtained from
the preceding maneuver, *i.e.* at pH 4.5, was taken up in water and

dissolved by addition of small amounts of dilute NaOH. Sufficient alcohol was added to bring it to a 70 per cent concentration

FIG 1. Photomicrograph of crystals of gastroglobulin obtained by the brucine-pyridine method.

Fig. 2   Fig. 3

FIG. 2. Photomicrograph of crystal of gastroglobulin obtained by crystallization at the isoelectric point.

FIG. 3. Photomicrograph of crystal of gastroglobulin obtained by crystallization by alcohol.

and the flask was placed in the ice box for several days. A small yield of beautifully formed crystals was found. These were not so well shaped as the others but approached the first type (see Fig. 3).

Studies on Filtrates and Dialysates

Magnesium Sulfate Filtrate—This filtrate was found to give a positive biuret reaction. Saturation of the solution with $MgSO_4$ or ammonium sulfate did not cause further precipitation. Likewise none occurred after boiling.

The contents of a series of tubes of gastric juice were adjusted to various hydrogen ion concentrations from pH 1.4 to 6.3. After

Fig. 4. Photomicrograph of the two main types of osazones obtained from the gastroglobulin-free acetic acid-acetone filtrate

they were brought to equal volume, they were one-half saturated with $MgSO_4$. The gastroglobulin was removed by filtration. The protein-like nitrogen content of each filtrate was calculated as the difference between the total nitrogen of each specimen minus the non-protein nitrogen determined upon the original gastric juice. It was found to be least in those tubes from which the most gastroglobulin had been removed; namely, at about pH 3.5 (see Table II).

Dialysis—Sufficient amounts of each filtrate as obtained above were dialyzed in separate celloidin bags until free from sulfates. Estimations for nitrogen were made upon the contents of each

sac (see Table II). It was found that filtrates which had been obtained at or about pH 3.5 were nitrogen-free. Away from this point more and more nitrogen was found. This nitrogen was from gastroglobulin which had not been precipitated in entirety at any pH except 3.5.

Acetic Acid-Acetone Filtrate—Many types of procedure were carried out on the filtrates from various mixtures. If a portion of

TABLE II

Mixture XIX. Results of Dialysis after One-Half Saturation of Gastric Juice with MgSO₄

The salting out was carried out at various hydrogen ion concentrations. NaOH was added to gastric juice to produce the desired pH; the contents were brought to unity. The contents of all tubes were then one-half saturated with $MgSO_4$.

pH as adjusted	Approximate pH of MgSO₄ mixture	Protein N per 100 cc gastric juice			
		Before dialysis		After dialysis	
		Filtrate	Precipitate	Filtrate	Precipitate
		mg	*mg.*	*mg.*	*mg.*
1 4	3 4	7 64	16 92	Blank	15 80
1 6	3 5	7 40	17 10	"	16 39
2 0	3 6	6 80	17 52	"	16 78
2 2	3 7	7 40	16 98	"	16 02
2 4	3 9	9 20	15 00	2 13	14 10
2 4	4 0	12 63	11 73	4 32	10 90
2 6	4 2	13 72	10 10		10 00
3 0	4 5	15 20	8 97		
3 4	4 6	16 44	8 10	6 93	7 85
3 5	4 8	17 32	7 19	7 68	7 00
4 0	5 4	17 60	6 92	7 73	6 53
4 6	5 8	17 70	6 92		
5 4	6 2	17 79	6 60		6 00
6 3	6 3	17 81	6 52	8.19	5 72

the material was placed in the ice box for several days, a small precipitate formed which, when removed, was found to give the same protein reactions as the main precipitate and did not reduce alkaline copper sulfate. Another portion, on boiling, showed some coagulum. This precipitate gave the same reactions as that obtained in the ice box. Furthermore, the nitrogen content of the two was approximately the same (the diagram shows the agreement in the two methods).

The filtrate when freed of these ice box or heat precipitates was found to give strongly positive reactions to the biuret, Hopkins-Cole, and xanthoproteic tests, but was consistently negative to Millon's test.   No precipitate was obtained by heating, $(NH_4)_2SO_4$, $MgSO_4$, alcohol, brucine, or pyridine.   All substances containing nitrogen were easily dialyzed through celloidin membranes which were impermeable to the gastroglobulin.   After digestion of the filtrate with 2 per cent H_2SO_4 in a water bath for 2 to 3 hours, a very real reduction of copper sulfate was produced.   At pH 3.5 a bulky precipitate was obtained by tungstic acid.   This precipitate, when redissolved, gave all the protein tests except Millon's, and contained quantitatively the nitrogen not accounted for by non-

TABLE III

Amount of Sugar in Relation to Protein Nitrogen

The results are expressed in mg. per 100 cc. of gastric juice.

Mixture No	Gastro-globulin N	Dialyzable protein N	Total protein N	Sugar expressed as glucose	
				Whole gastric juice	Dialyzable protein-like material
XVII	12 15	6 65	18 80	6 98	6 50
XVIII	22 14	11 87	35 01	14 28	12 25
XVIII-a	31 32	21 42	52 74	7.31	7 00
XIX	17 32	6.20	23 52	34 60	32.60
XXI	14 68	6 40	21 08	8 54	8 00

protein nitrogen; after hydrolysis it contained, also quantitatively, the $CuSO_4$-reducing substance.   Repeated tests for sugars were carried out on filtrates from different mixtures of gastric juice.

At times it was found that with bakers' yeast a small, slow, but definite fermentation took place.   The content of sugar was found to vary between 7 and 32 mg., expressed as glucose per 100 cc. of gastric juice.   In Table III a comparison is given of the two protein substances in relation to amounts of sugar and it may be seen that in this series of results no agreement could be found.

It was repeatedly noted that the amount of sugar liberated by prolonged hydrolysis of the complete gastric juice was identical with that liberated from the acetic acid-acetone filtrate after hydrolysis.   The same amount of sugar was found in the tungstic acid precipitate obtained from this filtrate at pH 3.5.

Osazones were made from the same specimens in which the amount of sugar was estimated. Two main types were found and are illustrated in Fig. 4. One consisted of long, thin, pointed, yellow needles which tended to arrange themselves in the fan-like arrangement typical of glucosazones. The other was found as compact, circular, yellow masses. The ends were pointed. At times the individual radials tended to be much broader than others. It is possible that there may be two types of osazones within this classification.

To simplify the study of the several methods of procedure and to collect the data under one schema so that the relation of the various compounds to each other and to the substances of the orig-

TABLE IV

Sugar Content of Gastric Mucus in Relation to Its Protein and Non-Protein Nitrogen Content

Mixture No.	N per 100 cc gastric juice			Sugar, as glucose, per 100 cc gastric juice	
	Total N	Non-protein N	Protein N	Mucus not dialyzed	Mucus dialyzed
	mg.	*mg*	*mg.*	*mg.*	*mg*
18	70 3	39 12	31 18	96 8	
19	68 24	35 31	32 93	86 48	72 22
22	82 2	43 37	38 83	60 0	55 4

inal gastric juice may be more readily checked, the protocol from one type of experiment is given in the accompanying diagram.

Mucus— Mucus from a known quantity of gastric juice as extracted was collected from the filter paper of three mixtures. After centrifugation the supernatant fluid was decanted and the mucus was mixed with sufficient NaOH to bring it to a homogeneous consistency. Total nitrogen, non-protein nitrogen, and sugar were determined and expressed in terms of 100 cc. of original gastric secretion; *i.e.*, the mixture of filtrable and non-filtrable material. Sugar was liberated only after hydrolysis in 2 per cent H_2SO_4 for 2 to 3 hours. The results are shown in Table IV.

It is quite evident that the sugar content of mucus is much higher in relation to protein than was the sugar of the acetic-acetone filtrate in relation to the protein-like nitrogen.

Filtered gastric juice

Protein N 23.52, non-protein N 35.8, sugar 34.8 (expressed as mg. per 100 cc. gastric juice)

Branch A — One-half saturated MgSO$_4$, at pH 3.5

Precipitate

Gastroglobulin N 17.32

Protein tests
Biuret +
Millon +
Xanthoproteic +
Hopkins-Cole +

Sugar tests after hydrolysis
All 0
CuSO$_4$ 0
Osazones 0
Fermentation 0

After dialysis
Gastroglobulin N 16.6
Protein sugar, as above
Isoelectric pH 3.4–3.6

Analysis
C 53.14
H 6.44
N 12.80
S 1.37
P 0
Ash 1.82

Filtrate

Protein N 6.8

Protein tests
Biuret + (Too much MgSO$_4$ for other tests)

After dialysis N 0

Branch B — 2 per cent acetic acid, 33 per cent acetone

Precipitate

Gastroglobulin N 15.08

Protein tests
Biuret +
Millon +
Xanthoproteic +
Hopkins-Cole +

Sugar tests after hydrolysis
All 0

After dialysis
Gastroglobulin N 14.3
Protein sugar, as above
Isoelectric pH 3.4–3.6

Analysis
C 51.83
H 7.21
N 11.48
S 1.76
P 0
Ash 2.35

Filtrate → **Ice box**

  Precipitate

  Gastroglobulin N 2.41

  Protein tests
  All 0

  Sugar tests after hydrolysis
  All 0

  Filtrate

  Protein N 6.0

  Protein tests
  Biuret +
  Millon 0
  Xanthoproteic +
  Hopkins-Cole +

  Sugar tests after hydrolysis
  CuSO$_4$ 32.02
  Osazones +
  Fermentation ±

  After dialysis N 0

Filtrate → **Water bath**

  Precipitate

  Gastroglobulin N 2.34

  Protein tests
  All +

  Sugar tests after hydrolysis
  All 0

  Filtrate

  Protein N 6.08

  Protein tests
  Biuret +
  Millon 0
  Xanthoproteic +
  Hopkins-Cole +

  Sugar tests after hydrolysis
  CuSO$_4$ 32.8
  Osazones +
  Fermentation ±

  After dialysis N 0

Filtrate + tungstic acid precipitation, pH 3.5

Precipitate

Redissolved pH 6.5
N 6.0

After hydrolysis
S. gar 31.4
Osazones +
Fermentation ±

Filtrate

N 35.7

After hydrolysis
CuSO$_4$ 0
Osazones 0
Fermentation 0

DISCUSSION

It has long been known that in the mucus of gastric juice there is a mucoprotein. It is reported (9) to be a protein to which are attached equimolecular amounts of glucuronic, acetic, and sulfuric acids and a carbohydrate. One of the characteristic properties of mucoproteins is their precipitability by dilute acetic acid.

In this study it has been shown that, in addition to the two protein-like bodies which have been described, there is, in the filtered, limpid gastric juice, a protein which is precipitated in small amounts at pH 6.8 to 7.0 and which contains sugar. It has not been obtained in sufficient quantities for more thorough study. It may be a mucoprotein. In this connection it is interesting that

the addition of acetic acid to the filtered, limpid gastric juice caused, at times, a very light precipitate. This precipitate is not to be confused with the heavy precipitate that appeared upon the addition of acetone to gastric juice which had been rendered 2 per cent with reference to acetic acid.

Some years ago Nencki and Sieber (10) reported some studies of the proteins of the gastric juice. They believed that they had demonstrated a nucleoprotein which was coagulated by heat. This, however, must have been due to improper washing of the precipitate. They also mention a dialyzable substance which they believed to be a proteose. It may be that the protein-like substance with the attached sugar molecule, reported above, is similar to the compound noted by them.

<div align="center">TABLE V</div>

<div align="center">*Chemical Analysis of Gastric Proteins Reported Recently*</div>

Elements	By Fenger, Andrew, and Ralston (12) from gastric mucosa of animals			By Northrop (7) from pepsin, u s p	By Webster and Komarov (11) from dog gastric juice	By Martin from human gastric juice	
	pH 2.5	pH 3 0	pH 3 8	pH 2 75		pH 3 5	pH 3 5
C...... .. .	53.15	52 27	51 49	52 40	52 59	53 14	51 83
H...... ..	7 02	6 93	7 03	6 67	7 01	6 44	7 21
N...	14 43	14 63	14 43	15 30	13 70	12 80	11 48
S............	0 88	0 96	1 08	0 86	1 29	1 37	1 76
P......	0 13	0 11	0 08	0 078	0 00	0 00	0 00
Cl	0 00	0 96	1 02				

Webster and Komarov (11) obtained gastric juice by histamine stimulation from fundic pouches of dogs. They added acetic acid to it to make a 1 per cent solution and subsequently $1\frac{1}{2}$ volumes of acetone. They obtained a heavy precipitate which gave positive reactions to the biuret, xanthoproteic, Hopkins-Cole, and Millon tests. After hydrolysis they obtained a sugar. The chemical analysis obtained by them is shown in Table V. They believed the substance to be a mucoprotein.

In 1928 Fenger, Andrew, and Ralston (12) obtained a protein from the gastric mucosa, which they identified as pepsin. It gave positive biuret, xanthoprotein, Hopkins-Cole, and Millon's tests. They showed that the substance could be precipitated from the mucosal extract at pH 2.5, 3.0, and 3.8. Greater peptic activity

was obtained when precipitation occurred at pH 2.5.   The chemical analysis of these three preparations is also shown in Table V.

Northrop in his recent article on crystalline pepsin reported its chemical composition, which is also found in Table V.   He found that its isoelectric point was pH 2.75.   His crystals gave positive biuret and xanthoproteic tests and were negative to the Molisch reaction.

The noteworthy difference shown in the comparison of these figures is the fact that much less nitrogen was found in the preparations obtained from the gastric juice than from the gastric mucosa. This would seem to make quite remote the possibility that gastroglobulin and pepsin were the same substance.   However, the similarity of the crystalline gastroglobulin obtained by the brucine method and the data reported in Paper II make complete separation at present uncertain.

In line with this discussion it may be mentioned that crystals of gastroglobulin, obtained without other agents and at the isoelectric point, resemble in shape those of urease as prepared by Sumner.   This fact is interesting in the light of the observations in Paper II.

SUMMARY

1. At pH 6.8 to 7 a small amount of protein containing sugar is precipitated from filtered, limpid, human gastric juice.

2. A protein has been obtained in crystalline form from gastric juice.   It is precipitated from gastric juice by one-half saturation with magnesium sulfate or ammonium sulfate and by a gastric juice rendered 2 per cent with reference to acetic acid and 33.3 per cent with reference to acetone.   It is coagulated by heat and is not dialyzable.   It has been called gastroglobulin.

3. There is in addition a protein-like material which cannot be precipitated by saturation with magnesium sulfate or ammonium sulfate.   It is not coagulated by heat and is acetone-soluble.   It is dialyzable.   It can be precipitated by tungstic acid at pH 3.5. It contains quantitatively all the sugar of the gastric juice not accounted for by the mucus.   Osazones have been obtained on the sugars.

4. Attention is drawn to the similarity of the shape of the gastroglobulin crystals to those of pepsin prepared by Northrop.   By

another method of precipitation crystals were obtained similar to those of Sumner's urease.

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GASTRIC JUICE

II. STUDIES ON A UREA-SPLITTING ENZYME AND PEPSIN IN RELATION TO THE PROTEINS

By LAY MARTIN

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It has been shown that a urea-splitting enzyme is present in normal gastric juice (1). Its range of activity was at least between pH 1.4 and 8.3, and its greatest action was found to take place at pH 7.8. Its activity was slow, and continued over a space of several days; the greatest part had taken place by the end of the first 24 hours. Owing to these characteristics it was suggested that the enzyme was probably different from that isolated and crystallized from the soy bean by Sumner (2).

In connection with the studies described in Paper I (3), it became of interest to determine to which of the two protein or protein-like substances of the gastric juice the urea-splitting enzyme and pepsin were attached.

EXPERIMENTAL

Unless specified, methods of procedure were identical to those used in Paper I (3).

Determination of Urea-Splitting Enzyme

Not Dialyzed—Mixtures of gastric juice containing free HCl were adjusted to various hydrogen ion concentrations from 1.4 to 6.3. After the contents of the tubes were made equal with water, they were one-half saturated with magnesium sulfate. The contents of the tubes were filtered through Whatman No. 42 filter paper, and the precipitate was redissolved in an amount of water equal to the original gastric juice specimen. Filtrates and redissolved precipitates were analyzed for nitrogen content. 10 cc.

portions of the contents of each tube were adjusted to pH 7.4 with phosphate buffer, and 2 cc. of a solution containing 7.04 mg. of urea nitrogen per 100 cc. were added to the filtrate. The original urea content of the filtrate was 2.52 mg. of nitrogen per 100 cc. To 10 cc. of the redissolved precipitate buffered as above, 2 cc. of a

TABLE I

Relation of Urea-Splitting Activity to Protein Nitrogen Salted Out at Various pH Values

Adjusted pH of gastric juice	Approximate pH of tubes after one-half saturation with MgSO$_4$	Not dialyzed				Dialyzed			
		Protein N per 100 cc gastric juice		NH$_3$-N after incubation with urea, 1 53 mg per cent		Protein N per 100 cc. gastric juice		NH$_3$-N after incubation with urea, 1.53 mg per cent	
		Precipitate	Filtrate	Precipitate	Filtrate	Precipitate	Filtrate	Precipitate	Filtrate
		mg	*mg*	*mg*	*mg*	*mg.*	*mg*	*mg*	*mg.*
1.4	3 4	16 92	7 64	0 23	Blank	14 80	Blank	0 30	Blank
1 6	3 5	17 10	7 40	0 22	"	15 10	"		"
2 0	3 6	17 32	7 20	0 23	"		"		"
2 2	3 7	16 98	7 40	0 23	0 08	14 50	"	0 24	"
2 4	3 9	15 00	9 20	0 22	0 17	12 00	2 13		0 16
2 5	4 1	11 23	12 63	0 22	0 12	10 60	4 13	0 18	0 24
2.6	4 2	11 10	13 72		0 10	10 00		0 17	
3 0	4 5	8 57	15 20	0 17	0 13			0 16	0 24
3 4	4 6	8 30	16 44	0 17	0 15		6 93		0 26
3 5	4 7	7 89	17 32	0 13	0 15		7 68		0 24
4 0	5.4	7 20	17 70	0 14	0 17	7 00	7 53	0.13	0 32
6 3	6 3	6 50	17 81	0 10	0 17	6 00			

N per 100 cc.
mg.

NH$_3$-N from gastric juice   buffered and incubated at pH 7.4 = 0.2
  "   "   gastroglobulin   "   "   "   "   " 7.4 = 0.0
  "   "   urea   "   "   "   "   " 7.4 = 0.0
  "   "   gastric juice   "   "   stored in ice box 7.4 = 0.0

solution containing 9.16 mg. of urea nitrogen per 100 cc. were added. This made a final urea nitrogen concentration of 1.53 mg. per cent in each tube of filtrate and precipitate, for at this time there were two series of tubes, one of filtrate and one of precipitate, which contained urea. To obtain controls, the contents of the tubes were halved. In this way it was possible to place similar preparations of each tube in the incubator at 37.5° and in the ice

box at 7.0° for 48 hours.   At the end of this period the contents of
the tubes were analyzed for ammonia nitrogen, urea nitrogen, and
ammonia and urea nitrogen *in toto*, as described in a previous article
(1).   The results are shown in Table I.

Dialyzed—A second series of tubes was identically arranged and
one-half saturated with magnesium sulfate.   After filtration the
precipitates were redissolved in an amount of water equal to the
amount of gastric juice from which they were obtained.   Filtrate
and precipitate were dialyzed in celloidin membranes prepared by
the method of Pierce, as previously described (3).   Dialysis was
continued until the dialysate was found free from sulfates.   As
shown in Paper I, only the gastroglobulin nitrogen remained within
the sacs.

The contents of the sacs were made equal with water, and a
portion of each was analyzed for nitrogen.   Another portion repre-
senting a known amount of nitrogen was adjusted to pH 7.4 with
phosphate buffer.   2 cc. of a solution containing 9.16 mg. of urea
nitrogen per 100 cc. were added to each tube and the contents were
brought to uniform volume, making a final urea nitrogen concen-
tration of 1.53 mg. per cent.   Toluene was added to each tube.
The contents of each tube were halved; one part was incubated at
37.5° for 48 hours; the other was placed in the ice box, temperature
7.0°.   At the end of this period the specimens were analyzed for
ammonia nitrogen, urea nitrogen, and total ammonia and urea
nitrogen, as previously described (1).   The increase of ammonia
nitrogen was quantitatively accounted for by the decrease in urea
nitrogen.   Controls were run with (*a*) the original gastric juice
incubated without the addition of urea, (*b*) gastroglobulin incu-
bated with addition of urea (a portion of this material was incu-
bated without urea), (*c*) urea blank, (*d*) the original gastric juice
which had been placed in the ice box.

In a previous article (1) it has been shown that ammonia pro-
duction occurred without demonstrable presence of bacteria.
Mixtures were cultured before and after incubation at 37.5°.

The results of a single experiment which were typical of the three
carried out are shown in Table I.

An analysis of the data demonstrates several points.   (1) The
non-dialyzed urea-splitting enzyme was found in connection with
the gastroglobulin and its greatest activity was slightly greater

than the activity of the original gastric juice. (2) The amount of ammonia formed was in direct relation to the amount of gastroglobulin. (3) After dialysis the urea-splitting enzyme tended to increase in activity and it was again in direct relation to the amount of gastroglobulin. (4) No ammonia was formed in any of the tubes placed in the ice box or where gastroglobulin or urea was

TABLE II

Relation of Peptic Activity to Protein Nitrogen Salted Out at Various pH Values

Adjusted pH of gastric juice	Approximate pH of tubes after one-half saturation with MgSO$_4$	Not dialyzed			Dialyzed			
		Protein N in mg per 100 cc. gastric juice		Non-protein N after incubation ½ hr at 37.5° with 1 per cent casein at pH 1 4	Protein N in mg. per 100 cc. gastric juice		Non-protein N after incubation ½ hr at 37.5° with 1 per cent casein at pH 1 4	
		Precip- itate	Fil- trate	Precipitate*	Precip- itate	Filtrate	Precip- itate	Filtrate
				mg.			*mg.*	*mg.*
1 4	3.4	16 92	7 64	19 10	14 80	Blank	16 97	Blank
1 6	3 5	17.10	7 40	20 20	15 10	"	17 32	"
2 0	3 6	17 32	7 20			"		"
2 2	3 7	16 98	7 40	19 98	14 50	"	16 02	"
2 4	3 9	15 00	9 20	16 88	12 00	2 13	12 42	Trace
2.5	4 1	11 23	12 63	12 48	10 60	4 13	12 00	4 23
2 6	4 2	11 10	13 72	11 49	10 00		11 32	
3.0	4 5	8 57	15 20	11 40				
3.4	4 6	8 30	16 44	11 01		6 93		6 33
3 5	4 7	7 89	17 32	9 66		7 68		7 74
4 0	5.4	7 20	17 70	9 21	7 00	7 53	8 80	8 21
6 3	6 3	6 50	17 81	9 05	6 00		7 70	

Non-protein N from peptic activity in gastric juice = 21 2 mg. per 100 cc.
  " " " casein control   = 0 0 " " 100 "
 * Not determined in filtrate owing to heavy MgSO$_4$ precipitate.

incubated separately. (5) The amounts of ammonia nitrogen formed and urea nitrogen destroyed were approximately equal.

Determination of Pepsin

A similar study was made on the enzyme pepsin. Peptic activity was measured by the amount of non-protein nitrogen

formed from a 2 per cent casein suspension, which had been mixed with 2 cc. of a 1 : 10 dilution of gastric juice or of an equal amount and dilution of the various filtrates and precipitates. The mixtures were incubated for $\frac{1}{2}$ hour in a water bath at 38°. The unit of measurement was expressed as mg. of non-protein nitrogen formed by the action of 100 cc. of original juice acting for $\frac{1}{2}$ hour on 2 per cent casein. Proteins were precipitated by the addition of 5 cc. of 20 per cent trichloroacetic acid. Nitrogen of the filtrate was determined by the method of Folin and Wu (4).

Peptic activity was determined on dialyzed and non-dialyzed materials similar to those used for the investigation of the urea-splitting enzyme activity. Owing to the large amounts of $MgSO_4$ in the non-dialyzed filtrate a determination could not be carried out on this portion.

The data are shown in Table II and demonstrate the following points: (1) Active pepsin was found only in connection with the gastroglobulin. (2) The activity of the dialyzed and non-dialyzed enzyme was in direct relation to the amount of gastroglobulin. (3) The peptic activity of the specimen precipitated at pH 3.5 was practically equal to that of the original gastric juice.

DISCUSSION

From the work reported it is shown that pepsin and a urea-splitting enzyme were found in close quantitative relation to the gastroglobulin of the gastric juice; so close in fact that one wonders if the enzymes do not form most, if not all, of the protein.

Various investigators have shown that the isoelectric point of pepsin obtained from the gastric mucosa is about pH 3.0. Northrop has shown that the pepsin which he has crystallized has pH 2.75 as its isoelectric point. The gastroglobulin described above has its isoelectric point at pH 3.5.

SUMMARY

Data have been presented to show that a urea-splitting enzyme and pepsin are quantitatively related to the gastroglobulin of the gastric juice. The urease activity was extremely weak and not comparable with that found in soy bean extract or in some bacterial cultures.

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CAROTENE

V. FORMATION OF GERONIC ACID BY OZONIZATION OF CARO-TENE, DIHYDROCAROTENE, AND RELATED COMPOUNDS

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INTRODUCTION

In order to gain further information concerning the structure of the carotene molecule the ozonization products of carotene have been investigated. Although the nature of these products was found to be dependent upon the conditions under which the ozonide was formed and decomposed, geronic acid, $CH_3 \cdot C{=}O \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot C \cdot (CH_3)_2 \cdot COOH$, was always present. Since the isolation of geronic acid determines the relative position of 9 carbon atoms in the carotene molecule, it became of interest to ascertain the maximum number of mols of geronic acid produced per mol of carotene ozonized. Accordingly a sensitive method for the determination of geronic acid as the 2,4-dinitrophenylhydrazone was developed and conditions of ozonization were altered until maximum yields of geronic acid were obtained. Depending on con-
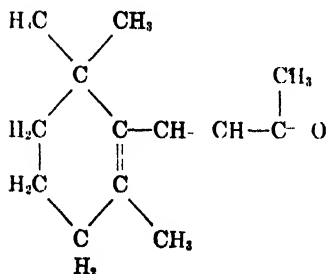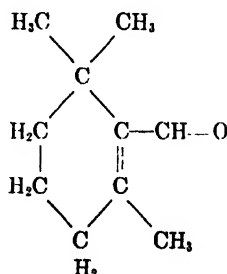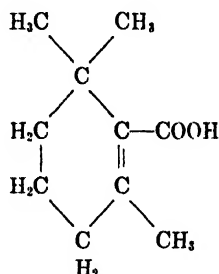
I. β-Ionone   II. β-Cyclocitral   III. β-Cyclogeranic acid

ditions, 0.25 to 0.67 mol of geronic acid was obtained in the form
of the recrystallized 2,4-dinitrophenylhydrazone per mol of caro-
tene ozonized. Similar yields of geronic acid were also obtained
from dihydrocarotene. Separate experiments with β-ionone
(Formula I), β-cyclocitral (Formula II), and β-cyclogeranic acid
(Formula III) demonstrated that, under the conditions employed
with carotene, 0.32 to 0.78 mol of geronic acid could be recovered
per mol of substance ozonized. The results of a number of such
experiments are summarized in Table I.

While this work was in progress, Pummerer and coworkers re-
ported that ozonization of carotene produced 0.16 mol of geronic
acid per mol of carotene ozonized (1). Karrer has also reported
that ozonization of carotene and dihydrocarotene produced 0.32
and 0.37 mol of geronic acid per mol of pigment respectively, while
ozonization of β-ionone produced only 0.194 mol of geronic acid
per mol of ketone ozonized when the geronic acid was isolated as
the crude semicarbazone. On the basis of these observations
Karrer and Morf concluded that carotene contained two β-ionone
groups which gave rise to the geronic acid (2).

Experience with ozonization methods has shown that the yield
of any given product varies within wide limits with slight changes
in the conditions under which the ozonide is formed and decom-
posed. Moreover, slight changes in the spacial relations and of
substituted groups are also known to affect the yields of the ozoniza-
tion products of many substances. Hence one is forced to con-
clude that calculations based upon a comparison of the yields of
ozonization products may be quite misleading. In the case of
carotene and dihydrocarotene no experimental results have yet
been obtained which establish the total number of groups that
form geronic acid when the molecules are decomposed with ozone.

Since both carotene (3) and dihydrocarotene exhibit the odor of
violets when exposed to the air it has been suggested that at least
one β-ionone group is present in each substance and that fission
of this group by ozone leads to the production of the geronic acid
(4, 5). The increase in the yields of geronic acid obtained by
decomposing the ozonides with hydrogen peroxide (see Table I,
Experiments 1, 3, 5, 7, 8, 9) also supports this hypothesis, for it is
well known that peroxides readily oxidize α-keto acids such as one

would expect as an intermediate ozonization product of a β-ionone or β-cyclocitral group as illustrated by Formulas IV to VI.

IV. β-Cyclocitral

V. Intermediate keto acid or ozonide
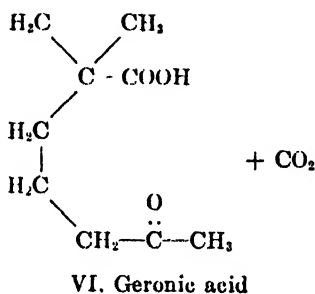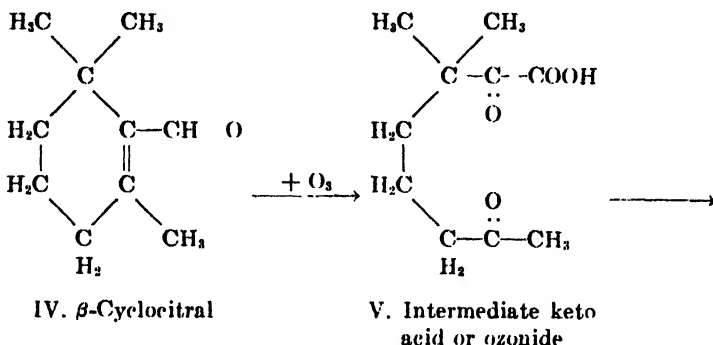
VI. Geronic acid

The present paper contains a description of (a) the preparation of pure substances for ozonization, (b) the preparation of geronic acid by ozonization of β-cyclocitral and the preparation of derivatives of geronic acid, (c) the conditions under which the ozonization of the different substances was performed, the method of analysis, and the results obtained, (d) the preparation of isogeronic acid and its derivatives by ozonization of α-ionone, (e) the formation of acetic and formic acids by the ozonization of carotene in the presence of water.

<div align="center">EXPERIMENTAL</div>

<div align="center">*Preparation of Pure Substances*</div>

Carotene and Dihydrocarotene—The carotenes used in this investigation were prepared from plant material as described by Smith (6). The carrot root carotene exhibited a slight optical

activity $[\alpha]_{6678}$ about $+40°$; m.p., corrected, 170°. The leaf carotenes were optically inactive; m.p., corrected, 180–181°. The dihydrocarotenes were prepared as described by Smith (7). When exposed to air in the presence of ether and alumina, the dihydrocarotenes produced a marked odor of violets.

β-Cyclocitral was prepared by condensing citralaniline with concentrated sulfuric acid as described by Haarmann and Reimer[1] (8) and by condensing citralcyanoacetic acid with sulfuric acid as described by Tiemann (9). Of these two methods the latter proved to be the more satisfactory. The crude β-cyclocitral was purified by means of the semicarbazone (10). The purified semicarbazone melted at 167.5°, corrected, as recorded by Tiemann. The β-cyclocitral was recovered by distilling the semicarbazone with phthalic anhydride in a current of steam.

When citralcyanoacetic acid was condensed with concentrated phosphoric acid and the products resolved by means of the semicarbazones, it was found that β-cyclocitral was the principal product of the reaction. This observation contradicts the hypothesis that condensation by phosphoric acid in the citral series should produce the α-cyclo compounds (11).

β-Cyclogeranic acid was prepared by the autoxidation of pure β-cyclocitral in air. The crystals were further purified by recrystallization from acetic acid and water. They melted at 93–94°, corrected (9).

β-Ionone was prepared by condensing pure β-cyclocitral with acetone in the presence of sodium ethylate as described by Tiemann (9). The β-ionone thus formed was converted into the semicarbazone and recrystallized from alcohol; m.p., 147–148°, corrected. The β-ionone was recovered from the purified semicarbazone by distilling the latter with steam in the presence of tartaric acid. It boiled at 115–116°, uncorrected, at 3 to 4 mm. pressure. The refractive index determined with an Abbe refractometer agreed with the value of $n_D^{17.0}$, 1.521, previously reported by von Auwers and Eisenlohr for β-ionone prepared from the semicarbazone (12). Found, n_D 13.0°, 1.5233; 14.0°, 1.5230; 15.0°, 1.5225; 18.0°, 1.5210; 20.0°, 1.5200; 23.0°, 1.5185; 25.0°, 1.5175; 28.0°, 1.5160.

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[1] Prepared by Dr. C. W. Niemann.

α-Ionone was prepared by condensing pseudoionone with phosphoric acid as described by Hibbert and Cannon (11). In order to eliminate all traces of β-ionone, the product was resolved two times with sodium bisulfite as described by Chuit, Naef and Company and by Haarmann and Reimer (13). The yields of α-ionone were comparable to those reported in the literature. The pure α-ionone boiled at 99–101° at 1 mm. pressure. Its refractive index (Abbe) was the same as that previously reported. Found n_D 16.0°, 1.5006; 20.0°, 1.4991; 23.0°, 1.4978; 25.0°, 1.4969. Recorded, n_D, 16.5°, 1.50048; 17.2°, 1.50001 ((13) p. 168); 22.3°, 1.4984 (12).

Geronic Acid and Derivatives

In order to perfect methods for the separation and identification of geronic acid, it was necessary to synthesize this material by standard methods and to study its properties. Of the methods described for the preparation of geronic acid, all are known to give small yields of the acid contaminated with large quantities of by-products (1, 2, 9, 10, 14, 15). Consequently it was decided to prepare geronic acid by the ozonization of β-cyclocitral, which should yield only geronic acid and carbon dioxide as was indicated in the introduction to this paper.

β-Cyclocitral was ozonized in 5 times its weight of glacial acetic acid at room temperature for 6 hours. Then an excess of water was added, and the solution was warmed to 50° for 1 hour and distilled with steam. The residue from the steam distillation was extracted with five 100 cc. portions of ether. The ether was evaporated and the residue distilled at 3 mm. pressure, b.p. 143–148°. From two 5 and one 8 gm. portions of β-cyclocitral there were obtained 53.0, 61.1, and 57.5 per cent yields of distilled geronic acid. The latter was identified by converting it into the semicarbazone, m.p. 161–162°, corrected; geronic acid semicarbazone melts at 164° (16).

$C_{10}H_{19}O_3N_3$.	Calculated.	N 18.34,	neutralization equivalent[2] 229
	Found.	" 18.54,	" " 233
		" 18.37,	" " 234

[2] The neutralization equivalents were determined by dissolving 5 to 15 mg. of the substance in 4 cc. of alcohol, neutralizing to bromothymol

Derivatives of geronic acid and the common hydrazines were found to be slightly soluble oils or sticky crystals. Of the twelve hydrazines tested only 2,4-dinitrophenylhydrazine formed a derivative suitable for identification purposes. The condensation products of geronic acid and aromatic aldehydes, although only slightly soluble in dilute acids, were unsuited for the determination of geronic acid because none of them appeared to have a constant composition.

Geronic acid 2,4-dinitrophenylhydrazone was prepared by treating an aqueous solution of geronic acid with a solution of 2,4-dinitrophenylhydrazine in dilute hydrochloric acid. Portions of the precipitate were recrystallized several times from acetic acid and water, from methyl alcohol and water, and from benzene. Each portion melted at 135.5–137°, corrected. Analysis gave the following results.[2, 3]

$C_{16}H_{20}O_6N_4$.   Calculated.   N 15 91, neutralization equivalent 352
                      Found.   " 16 08,   "   "   362
                                     " 16.18,   "   "   349
                                     " 16 02

It was found that geronic acid could be recovered from the 2,4-dinitrophenylhydrazone by heating the latter with glyoxal sodium bisulfite or with methylglyoxal in dilute acid solution. Thus from finely ground geronic acid 2,4-dinitrophenylhydrazone (1.26 gm.) heated with a slight excess of glyoxal sodium bisulfite and dilute acetic acid for 10 hours, cooled, filtered, and extracted with ether there was recovered 0.35 gm. of geronic acid, b.p. 143–150°

blue with 0.01 N sodium hydroxide, diluting with 25 cc. of water, warming, and titrating to a suitable end-point. Clift and Cook (17) have also used bromothymol blue as an indicator for the titration of the 2,4-dinitrophenylhydrazones of keto acids.

[3] When the 2,4-dinitrophenylhydrazones reported in this and the following paper were analyzed for carbon and hydrogen by the method of Pregl, the values for carbon were found to be 0.5 to 0.9 per cent too high. This appeared to be due to the non-absorption of nitric oxides by the lead peroxide, for compounds containing no nitro groups gave very satisfactory results, while substances such as 3,5-dinitrobenzoic acid, picric acid, and carefully purified 2,4-dinitrophenylhydrazones gave consistently high results for carbon. Consequently no values are reported for carbon and hydrogen.

at 3 mm. The recovered acid formed the characteristic semicarbazone, m.p. 161–162°.

$C_{10}H_{19}O_2N_3$. Calculated, N 18 34; found, N 18.45

Geronic acid 2,4-dinitrophenylhydrazone was split in a much shorter time when a solution in glacial acetic acid was treated with glyoxal sodium bisulfite. Addition of concentrated hydrochloric acid increased the speed of the reaction but for preparative purposes its use was unnecessary.

Geronic acid 2,4-dinitrophenylhydrazone is only slightly soluble in 1 per cent sulfuric and hydrochloric acids (approximately 0.03 gm. per liter at 18° and 0.09 gm. per liter at 60°). It is very soluble in sodium hydroxide and sodium carbonate solutions but is rapidly decomposed by these reagents, especially upon warming. The hydrazone is also soluble in sodium bicarbonate solutions but is more stable to this reagent. Geronic acid 2,4-dinitrophenylhydrazone (0.2247 gm.) was dissolved in 50 cc. of sodium bicarbonate solution (4.4 per cent) and warmed on the water bath for $\frac{1}{2}$ hour. The solution was then acidified with sulfuric acid, cooled, and filtered. Weight of crystals 0.2117 gm. Per cent recovered 94.2. M.p. 123–134°, corrected. Recrystallized from acetic acid by the addition of water, the derivative melted at 133–134°, corrected. Analysis of a similar preparation demonstrated that the composition of the geronic acid 2,4-dinitrophenylhydrazone had not been altered.

$C_{15}H_{20}O_6N_4$. Calculated. N 15 91, neutralization equivalent 352
         Found.   " 15 88,   "   "   356, 358

Geronic acid 2,4-dinitrophenylhydrazone is slightly soluble in low boiling petroleum ether, soluble in acetic acid, acetone, benzene, n-butyl alcohol, ethyl ether, methyl alcohol, propylene glycol, and very soluble in carbon tetrachloride, nitrobenzene, ethyl acetate, and pyridine.

The 2,4-dinitrophenylhydrazones of glyoxylic, pyruvic, acetoacetic, and levulinic acids were also found to be decomposed by carbonate and stronger alkaline solutions, a reaction not recorded by other investigators (18). In general, keto acids may be separated and identified as the 2,4-dinitrophenylhydrazones as described for geronic acid.

Formation of Geronic Acid by Ozonization of Carotene and Related Compounds

The substance to be ozonized was placed in a 125 cc. Claisen flask and dissolved by the addition of carbon tetrachloride. The solution was cooled to 0° and treated with a moderately rapid stream of oxygen containing about 4 per cent of ozone. After some time (see Table I, Columns 5 and 6) acetic acid was added to the solution and the ozonization continued for several hours (Column 7). The ozonide was then decomposed by the addition of water or hydrogen peroxide (superoxol). After standing overnight the mixture was distilled with steam until about 50 cc. of distillate had been collected. The residue from the steam distillation was treated with a 10 per cent solution of 2,4-dinitrophenylhydrazine in 6 N sulfuric acid. The resulting mixture was permitted to stand 16 hours at 0°. It was then decanted through a Jena fritted glass filter and the residue washed with water. The precipitate was extracted on the filter with sodium bicarbonate solution (3 per cent), 3 cc. at 40°; 3 cc. at 60°; 3 cc. at 80°; then with four or five 5 cc. portions of 0.5 per cent sodium bicarbonate solution at 60–80°, and finally with water (10 cc.). Each extraction was made in 3 to 5 minutes. The geronic acid 2,4-dinitrophenylhydrazone was precipitated by acidifying the bicarbonate extract with dilute sulfuric acid. After standing 16 hours at 5° the precipitate was collected on a Jena fritted glass filter, washed, and dried in a desiccator. The geronic acid derivative was then dissolved in cold glacial acetic acid which was drawn through the filter. Addition of water (50 to 60 cc.) to the acetic acid caused the crystallization of the geronic acid 2,4-dinitrophenylhydrazone. In order that crystallization might be complete, the solution was permitted to stand in the refrigerator at 5° for 16 hours. The crystals were collected on a tared Jena fritted glass filter, dried in a vacuum over calcium chloride, and weighed. The results of these experiments are summarized in Table I.

Separate experiments demonstrated that the 2,4-dinitrophenylhydrazones of formaldehyde, acetaldehyde, acetone, acetol, glyoxal, methylglyoxal, and diacetyl could not have contaminated the geronic acid derivative, for the former were found to be insoluble in sodium bicarbonate solutions. Although the 2,4-dinitrophenylhydrazones of glyoxylic, pyruvic, acetoacetic, and levulinic acids

TABLE I

Yields of Geronic Acid Obtained by Ozonization of Carotene, Dihydrocarotene, and Related Compounds

Experiment No.	Substance	Amount	First ozonization		Second ozonization		Geronic acid 2,4-dinitrophenylhydrazone					Geronic acid per mol substance
			CCl_4	Time	Acetic acid	Time	Weight	M p., corrected	Mixed m p., corrected	Neutralization equivalent*	N†	
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)
		gm.	*cc*	*hrs*	*cc*	*hrs*	*gm*	*°C*	*°C.*		*per cent*	*mol*
1	β-Ionone†	0 1085	3 0	1 0	4 0	1 0	0 1211	134	134	360, 363	16 18	0 61
2	" §	0 1144	3 0	1 0	4 0	1 0	0 1030	133 5	134	352, 353	15 53	0 48
3	" semicarbazone‡	0 1216	3 0	0 0	4 0	2 0	0 0646	132 5	133	348, 348	16 28	0 38
4	" §	0 1169	3 0	0 0	4 0	2 0	0 0476	134 5	134 5	357, 367	15 85	0 29
5	β-Cyclogeranic acid†	0 1046	3 0	1 0	4 0	1 0	0 1707	135	135	360	16 05	0 78
6	" §	0 1004	3 0	1 0	4 0	1 0	0 0664	100	108	355, 360	16 29	0 32
7	Sunflower leaf carotene†	0 2211	9 0	1 0	11 0	1 5	0 0741	128	129	356, 364	16 12	0 51
8	Carrot root carotene‡	0 1600	6 0	1 0	8 0	1 0	0 0095	132 5	133	355, 352	16 09	0 67
9	" " †	0 1654	6 0	1 0	8 0	1 0	0 0655	131 5	132	349, 356	15 98	0 60
10	" " ‖	0 1524	6 0	0 75	6 0	1 25	0 0245	125	129	363	16 02	0 25
11	" " ¶	0 4800	25 0	1 0	25 0	2 0	0 1288	132 5	133 5	362, 366	16 18	0 41
12	" " ¶	0 5812	20 0	0 75	20 0	2 25	0 2159	133 5	133 5	355, 361	16 22	0 57
13	Dihydro carrot root carotene ¶	0 950	20 0	0 75	20 0	2 25	0 3382	135 5	135 5	361, 355	16 05	0 54

* Calculated 352.
† Calculated for $C_{15}H_{20}O_6N_4$, N 15.91.
‡ Ozonide decomposed with superoxol, 0 25 cc.
§ Ozonide decomposed with water, 0.20 cc.
‖ Ozonide decomposed with water, 2.00 cc.
¶ Ozonide decomposed with water, 5.00 cc.

are soluble in sodium bicarbonate solutions, these compounds were found to be much less soluble in cold glacial acetic acid. No indication of their presence was found when the geronic acid derivative was dissolved in acetic acid. Further proof of the absence of keto acids other than geronic among the ozonization products of carotene was obtained by extracting the geronic acid with small portions of ether and analyzing the residue with 2,4-dinitrophenylhydrazine. In such experiments no bicarbonate-soluble hydrazone was ever obtained.

Isogeronic Acid and Derivatives

Isogeronic acid was prepared by the ozonization of α-ionone. *A priori*, one should expect this reaction to take the course shown by Formulas VII to IX.
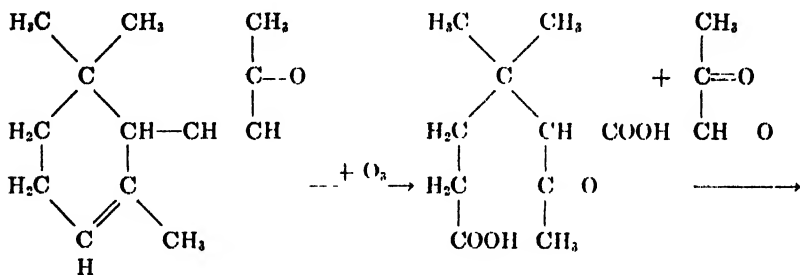
VII. α-Ionone   VIII. Intermediate keto acid
                                         (or ozonide)
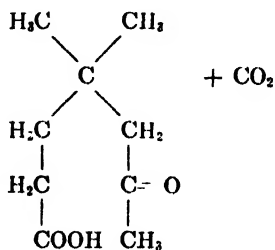
IX. Isogeronic acid

However, the low yields of isogeronic acid (5 to 8 per cent) isolated from the reaction products indicate that other reactions predominate. These results have an important bearing on the

structure of carotene, for the absence of isogeronic acid among the ozonization products of an optically active carotene may be due to the small yields of this acid rather than to the absence of an α-ionone ring (19, 20).

α-Ionone (4.13 gm.) was ozonized in acetic acid at 15° for $3\frac{1}{2}$ hours, decomposed with water and steam, and the residue treated with an excess of 2,4-dinitrophenylhydrazine solution which quickly formed a large quantity of tar-like material. The tar was soluble in bicarbonate solutions from which it was precipitated by the addition of acids. It was soluble in alcohol and acetic acid but separated as tar when the solutions were diluted with water. Finally the tar was dried, dissolved in acetic acid, and treated with petroleum ether (b.p. 50°). This solution deposited orange-yellow crystals (0.20 gm.). Per cent yield 3.8. After recrystallization from acetic acid and water and from acetic acid and petroleum ether, the crystals melted at 139.5–140°, corrected. They were identified as isogeronic acid 2,4-dinitrophenylhydrazone by analysis.

$C_{16}H_{20}O_6N_4$. Calculated. N 15.91, neutralization equivalent 352
Found. " 15 83, " " 346, 359

In another experiment α-ionone (4.57 gm.) was ozonized in carbon tetrachloride (25 cc.) at 0° for 2 hours. After standing overnight with water (3 cc.), the solution was distilled with steam The non-volatile material was extracted with ether and distilled at reduced pressure. Yield 0.8 gm.; b.p. 140–145° at 1 mm. A portion of this distillate formed a crystalline semicarbazone, m.p. 192–194°. Per cent yield 8.4. Recrystallized from acetic acid, the semicarbazone melted at 193–195°.

$C_{10}H_{19}O_3N_3$
Calculated. C 52 40, H 8 30, N 18 34, neutralization equivalent 229
Found. " 52.78, " 8 37, " 18 22, " " 225
" 52.85, " 8.44, " " 227

A second portion of the distilled acid formed a precipitate with a solution of 2,4-dinitrophenylhydrazine, m.p. 124–129°. Per cent yield 5.1. Recrystallized successively from acetic acid and petroleum ether, from acetic acid and water, from alcohol and water, from acetic acid, and from acetic acid and petroleum ether, orange-yellow crystals of isogeronic acid 2,4-dinitrophenylhydrazone were obtained which melted at 140–141°, corrected.

$C_{16}H_{20}O_6N_4$. Calculated, N 15 91; found, N 15 90

Isogeronic acid 2,4-dinitrophenylhydrazone is very similar to the corresponding hydrazone of geronic acid in regard to its solubility in water, alkalies, acids, and organic solvents.   This hydrazone is also decomposed by strong alkalies.   When mixed with geronic acid 2,4-dinitrophenylhydrazone or the 2,4-dinitrophenylhydrazones of other keto acids, the melting point was decidedly lower.

Formation of Acetic and Formic Acids by Ozonization of Carotene

Pummerer and coworkers (1) state that among the ozonization products of carotene they found only small quantities of glyoxal but no methylglyoxal.   An examination of the ozonization products of carotene by means of *m*-nitrobenzohydrazine carried out in this laboratory has confirmed their observations.   Although these results might appear to contradict the hypothesis that carotene contains a conjugated series of dehydrogenated isoprene groups, it is well known that many groups give rise to acids when the molecules are decomposed with ozone.

In order to determine the nature of the acids produced when carotene is ozonized, an apparatus was designed in which a suspension of carotene, carbon tetrachloride, and water could be treated with ozone at 0°.   Under these conditions it was found that large quantities of acetic and formic acids were produced and that the ratio of acetic to formic acid was $1.0:3.3$.   Ozonization of benzene under similar conditions demonstrated that formic acid was produced as the only volatile acid.   Ozonization of mesitylene produced acetic and formic acids in the ratio of $1.0:1.19$. These observations support the view that carotene contains dehydrogenated isoprene groups which are decomposed by ozone to form acetic and formic acids rather than glyoxal and methylglyoxal as shown by the following equation.

$$CH_3$$
$$\vert$$
$$=C-CH=CH-CH= + (O_3) + (H_2O) \rightarrow CH_3COOH + 3HCOOH$$
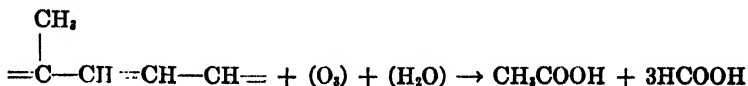
SUMMARY

Ozonization of carotene isolated from carrot roots and sunflower leaves produced nearly the same yields of geronic acid, 0.50 to 0.67 mol per mol of carotene.   The yield of geronic acid was not

changed by adding 2 atoms of hydrogen to carrot root carotene with aluminum amalgam.

Geronic acid was synthesized by ozonization of β-cyclocitral and β-cyclogeranic acid. Its 2,4-dinitrophenylhydrazone is described.

Condensation of citralcyanoacetic acid with concentrated phosphoric acid was found to produce β-cyclocitral rather than α-cyclocitral.

By making use of the slight solubility of geronic acid 2,4-dinitrophenylhydrazone, a method has been devised for the determination of geronic acid and other keto acids.

Glyoxal and methylglyoxal were used to split the dinitrophenylhydrazone of geronic acid.

Isogeronic acid was synthesized by ozonization of α-ionone. Its 2,4-dinitrophenylhydrazone was also prepared.

The 2,4-dinitrophenylhydrazones of keto acids were found to be very unstable towards alkalies.

The writer is greatly indebted to Dr. H. A. Spoehr, Director of this laboratory, and to Dr. J. H. C. Smith for many helpful suggestions. Mr. Harold W. Milner carried out the elementary microanalyses reported in this and the following paper.

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OZONIZATION OF LYCOPENE

FORMATION OF LEVULINIC ACID AND LEVULINIC ALDEHYDE

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Karrer and Bachmann reported that ozonization of lycopene produced an acid which gave, in addition to the iodoform reaction, the sodium-nitroprusside reaction characteristic of levulinic acid (1). Kuhn and Grundmann have since demonstrated that careful oxidation of lycopene produces methylheptenone, $(CH_3)_2 \cdot C = CH \cdot CH_2 \cdot CH_2 \cdot C \quad O \cdot CH_3$, and lycopenal, $C_{32}H_{32}O$ (2), and that oxidation of lycopene produces methylheptenone although the yields were very small (about 5 per cent) (3). Additional evidence that lycopene contains the group characteristic of levulinic acid has also been obtained in this laboratory where it has been shown that both levulinic acid and levulinic aldehyde are produced by ozonization of lycopene. Under suitable conditions 0.90 to 1.32 mol of levulinic acid were isolated as the 2,4-dinitrophenylhydrazone per mol of lycopene ozonized. These results substantiate the formula suggested for lycopene by Karrer and Bachmann (1).

Identification of Levulinic Acid—After experimenting with a number of hydrazine compounds, it was found that 2,4-dinitrophenylhydrazine was especially suited for the determination of levulinic acid. Levulinic acid synthesized from sucrose (4) or from citral (5–11) formed orange-yellow crystals of the 2,4-dinitrophenylhydrazone when treated with a solution of 2,4-dinitrophenylhydrazine in dilute acid. After recrystallization from acetic acid and water,[1] from nitrobenzene, ethyl acetate, and petroleum

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[1] It was found that large crystals of levulinic acid 2,4-dinitrophenylhydrazone obtained from a glacial acetic acid solution always contained some acetic acid which was extremely difficult to remove and which lowered

ether, and from alcohol, the levulinic acid 2,4-dinitrophenyl-hydrazone melted at 203–205.5°, corrected. The melting point previously recorded was 203° (12). When mixed with the 2,4-dinitrophenylhydrazones of glyoxylic, pyruvic, acetoacetic, geronic, and isogeronic acids, the levulinic acid derivative was found to melt at a much lower temperature.

$C_{11}H_{12}O_5N_4$.   Calculated.   N 18.92, neutralization equivalent 296
                     Found.   " 18.87,   "   "   294, 293

The *β-naphthylhydrazone* of levulinic acid proved to be a white crystalline compound, soluble in sodium bicarbonate solutions, and but slightly soluble in dilute acids. Recrystallized from ethyl acetate by the addition of petroleum ether the β-naphthylhydrazone melted at 136.5°, corrected. In time the crystals slowly turned red.

$C_{15}H_{16}O_2N_2$.   Calculated.   N 10 94, neutralization equivalent 256
                     Found.   " 10 75,   "   "   256, 260

Identification of Levulinic Aldehyde—An aqueous solution of levulinic aldehyde reacts with 2,4-dinitrophenylhydrazine to form the slightly soluble bis-2,4-dinitrophenylhydrazone. Repeated crystallization of this hydrazone from nitrobenzene produced yellow crystals, m.p. 235.5–236.5° corrected.

$C_{17}H_{16}O_8N_8$.   Calculated, N 24.35; found, N 24.33, 24.31

Levulinic aldehyde bis-2,4-dinitrophenylhydrazone is very slightly soluble in sodium bicarbonate solutions, in aqueous acids, and in the common organic solvents. It is quite soluble in hot nitrobenzene but only slightly soluble in the cold.

Levulinic Aldehyde from Lycopene—Lycopene (0.2050 gm.) isolated from tomatoes as described by Smith (13) was dissolved in a mixture of acetic acid (1 cc.), valeric acid (2 cc.), and carbon tetrachloride (2 cc.). The solution was cooled to 0° and ozonized for 5 hours; the red color disappeared after 20 minutes. The ozonide was permitted to stand 16 hours with water (0.1 cc.); it was

the neutralization equivalent from 296 to 270 to 276. Smaller crystals obtained from acetic acid and water contained less acetic acid and this was much more easily removed by drying the crystals over sodium hydroxide for several hours at 100° and 1 mm. pressure.

then distilled with steam and the distillate treated with a solution of 2,4-dinitrophenylhydrazine in dilute sulfuric acid.   The precipitate was filtered off and washed with glacial acetic acid.   The insoluble portion was recrystallized from nitrobenzene.   Weight of levulinic aldehyde bis-2,4-dinitrophenylhydrazone, 0.0529 gm. M.p., 232-233°, corrected; mixed m.p. with authentic levulinic aldehyde bis-2,4-dinitrophenylhydrazone, 233°, corrected.

$C_{17}H_{16}O_8N_8$.   Calculated, N 24.35; found, N 24 29, 24 24

In order to compare the ozonization products of citral with those of lycopene, citral (0.1200 gm.) was ozonized in carbon tetrachloride (1 cc.) and a mixture of acetic and valeric acids (1:1; 1 cc.). The ozonide was decomposed with water and analyzed with 2,4-dinitrophenylhydrazine as described for lycopene.   The recrystallized levulinic aldehyde bis-2,4-dinitrophenylhydrazone weighed 0.0574 gm.; m.p. 233.5°, corrected; mixed m.p. 233.5°, corrected.

$C_{17}H_{16}O_8N_8$.   Calculated, N 24 35; found, N 23 58

Levulinic Acid from Lycopene—Lycopene (0.2032 gm.) was suspended in carbon tetrachloride (6 cc.), cooled to 0°, and ozonized for 1 hour.   Acetic acid (8 cc.) was added and the ozonization continued $1\frac{1}{2}$ hours.   Nearly 2 hours were required to dissolve the lycopene during the ozonization.   The ozonide was decomposed with superoxol (0.25 cc.) over a 16 hour period at room temperature.   The excess acids were removed by distilling with steam and the residue treated with an excess of 2,4-dinitrophenylhydrazine dissolved in 6 N sulfuric acid.   After standing 16 hours at 5°, the solution was filtered, and the precipitate washed with water and extracted with 3 per cent sodium bicarbonate solution. The bicarbonate extract was acidified with sulfuric acid and permitted to stand 16 hours at 5°.   The precipitate was then filtered off and dried in a vacuum over calcium chloride.   It weighed 0.1485 gm.; m.p. 182–187°, mixed m.p. with authentic levulinic acid 2,4-dinitrophenylhydrazone, 185–188°.   This corresponds to a yield of 1.3 mols of levulinic acid per mol of lycopene ozonized. After recrystallization from acetic acid by the addition of water the derivative melted at 187–190°, corrected; mixed melting point, 190–192°, corrected.   Weight, 0.1071 gm.   This corresponds to a yield of 0.96 mol of levulinic acid per mol of lycopene.

$C_{11}H_{12}O_6N_4$. Calculated. N 18 92, neutralization equivalent 296
              Found.   " 18 94,   "   "   283

In another experiment, lycopene (0.1990 gm.) was dissolved in carbon tetrachloride (10 cc.) which was then cooled to 0° and treated immediately with ozone. After 25 minutes the lycopene was completely decomposed and the solution was filled with a solid ozonide. Acetic acid (8 cc.) was added and the ozonization continued 2 hours and 35 minutes. The clear and colorless solution was decomposed with superoxol (0.3 cc.) for 16 hours and analyzed as described above. Weight of sodium bicarbonate-soluble material, 0.1440 gm., m.p., 192- 194°, corrected, corresponding to a yield of 1.31 mols of levulinic acid per mol of lycopene. After recrystallization of the hydrazone from acetic acid and water it melted at 198–200°, corrected; mixed m.p. with authentic levulinic acid 2,4-dinitrophenylhydrazone, 199 201°, corrected. It weighed 0.1002 gm., which corresponds to a yield of 0.91 mol of levulinic acid per mol of lycopene.

$C_{11}H_{12}O_6N_4$. Calculated. N 18 92, neutralization equivalent 296
              Found.   " 18 94,   "   "   294

When citral (0.1070 gm.) was ozonized under the same conditions employed with lycopene, 0.1142 gm. of levulinic acid 2,4-dinitrophenylhydrazone was recovered, m.p. 192–194°, corrected, corresponding to a yield of 0.55 mol of levulinic acid per mol of citral. After recrystallization of the hydrazone from acetic acid and water, it weighed 0.0957 gm.; m.p. 202–203°, corrected. This corresponds to a yield of 0.46 mol of levulinic acid per mol of citral.

$C_{11}H_{12}O_6N_4$. Calculated. N 18 92, neutralization equivalent 296
              Found.   " 18 78,   "   "   293, 295

Ozonization of Xanthophyll—In order to determine whether ozonization of xanthophyll produces keto acids, xanthophyll isolated from spinach and from sunflower leaves was ozonized in a mixture of acetic acid and carbon tetrachloride. The ozonide was decomposed with superoxol, distilled with steam, and the residue analyzed with 2,4-dinitrophenylhydrazine as described for lycopene. In these experiments no alkali-soluble hydrazone was obtained. This indicates that keto acids other than the α or β isomers are not produced by the ozonization of xanthophyll.

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CAROTENE

VI. A NOTE ON THE HYDROGENATION OF α- AND β-CAROTENES

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In 1928 Zechmeister, von Cholnoky, and Vrabély (1) reported that carotene, on catalytic hydrogenation in cyclohexane solution, absorbs 11 mols of hydrogen per mol of pigment. Later the author (2) found that carotenes from different sources, in p-menthane-acetic acid solution, absorb only 10 mols of hydrogen. Recently Zechmeister, von Cholnoky, and Vrabély (3) have redetermined the hydrogenation ratio of carotene and proved beyond question that, in acetic acid solution, carotene absorbs 11 mols of hydrogen, but state, "bei der Anwendung von Menthan oder Dekalin als Lösungsmittel der Wasserstoff-Verbrauch nicht wesentlich über 10 H₂ zu treiben war." Because of this discrepancy the hydrogenation of carotene has been reinvestigated in this laboratory.

<center>EXPERIMENTAL</center>

Hydrogenation in Glacial Acetic Acid—The hydrogenation of carotene was carried out in glacial acetic acid so as to conform to the conditions used by Zechmeister and his associates. Samples of both α- and β-carotene were hydrogenated in order to determine whether the nature of the carotene had any effect on the hydrogenation ratio obtained.

Hydrogenation of β-Carotene (Carotene 1, Table I)—Carotene was obtained from the leaves of *Helianthus annuus*, L., by the method described in a previous paper (2). This carotene possessed a high melting point and was optically inactive.

$$[\alpha]_{6678} = \frac{0.00 \times 15.0}{1.0 \times 0.0444} = 0.0°$$

Because of these properties it was assumed to be β-carotene.

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After recrystallization from n-heptane it melted at 180° (Berl block) and gave the following analysis.

$$C_{40}H_{56}. \quad \text{Calculated.} \quad C\ 89\ 48,\ H\ 10.52$$
$$\text{Found.} \qquad \text{``}\ 89.43,\ \text{``}\ 10\ 57$$

This carotene was hydrogenated in the microhydrogenation apparatus by the method previously described (2). The reagents used were platinic oxide catalyst (4), recrystallized glacial acetic acid, and hydrogen from a cylinder. The data and results are given in Table I, Experiments 1 and 2. From these data it is concluded that the hydrogenation ratio of β-carotene is 11.

TABLE I

Results of Catalytic Hydrogenation of α- and β-Carotenes

Experiment No.	Carotene* No.	Weight of carotene	Weight of catalyst	Temperature	Barometric pressure	Volume of hydrogen absorbed	Hydrogen ratio observed	Solvent† No.
		mg.	*mg.*	*°C.*	*mm.*	*cc.*		
1	1	2 933	37 495	20 0	757 2	1 420	10 75	1
2	1	4.162	40 724	19 0	760 3	2 000	10 75	1
3	2	3.922	43 363	19 0	758 0	1 930	10 98	1
4	1	3 346	43 660	19 0	764 5	1 640	11 03	2
5	3	3 911	31 947	19 5	763 0	1 880	10 78	2

* Carotene 1 was β-carotene from *Helianthus annuus*, L.; Carotene 2, α-carotene from *Daucus carota*, L.; Carotene 3, from *Beta vulgaris*, L., var. *cicla*, L.

† Solvent 1 was 5.0 cc. of recrystallized glacial acetic acid; Solvent 2, 5.0 cc. of p-menthane-glacial acetic acid.

Hydrogenation of α-Carotene (Carotene 2, Table I) α-Carotene was prepared from carrot root carotene by the method described by Kuhn and Brockmann (5). In brief this method consists in adsorbing the β-carotene on fullers' earth from a petroleum ether solution of carrot root carotene and recovering the non-adsorbed fraction remaining in solution, which consists largely of α-carotene. The non-adsorbed fraction was crystallized several times from n-heptane. Only 0.037 gm. of α-carotene were obtained from 1.3 gm. of carrot root carotene. This material had the following properties:

$$C_{40}H_{56}. \quad \text{Calculated.} \quad C\ 89\ 48,\ H\ 10\ 52$$
$$\text{Found.} \qquad \text{``}\ 89.45,\ \text{``}\ 10.58$$
$$\text{M.p., 178.4° (Berl block)}$$

Specific rotation,

$$[\alpha]_{6678}^{19} = \frac{+\ 0.763 \times 15.0}{1\ 0 \times 0.0364} = +\ 314°$$

This carotene was hydrogenated in exactly the same way as the β-carotene and also was shown to possess a hydrogenation ratio of 11 (Table I, Experiment 3).

These results indicate that the hydrogenation ratios of α-carotene and of β-carotene are the same and that the differences in the hydrogenation results previously reported are probably not due to differences in the degree of unsaturation of the carotenes used.

Hydrogenation in p-Menthane and Acetic Acid—Hydrogenations of carotene were carried out in p-menthane-glacial acetic acid mixtures in order to repeat the conditions used in the previous experiments (2). The results of these experiments showed that the hydrogenation ratio was influenced by the condition of the solvent. First, when the solvent was contaminated by contact with a rubber stopper, a very low hydrogenation ratio, 7.49, was obtained.[1] Secondly, it was found that unless great care was exercised in the preparation of the solvent, the 11th mol of hydrogen was added exceedingly slowly and that a much longer time than was used previously must be allowed to complete the hydrogenation. Thirdly, it was observed that under some conditions, not clearly defined, there appears to be a liberation of hydrogen from the p-menthane-acetic acid solvent mixture. When this happens the apparent hydrogenation ratio will be too low because it will be the resultant of two reaction rates, the rate of absorption of hydrogen by carotene and the rate of liberation of hydrogen from solvent.

Finally a preparation of p-menthane-acetic acid was obtained in which the hydrogenation of carotene proceeded very rapidly and the hydrogen ratio of 10 was quickly exceeded. In this solvent a final value of 11.03 was attained (Table I, Experiment

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[1] The behavior of this solvent when it had come in contact with a rubber stopper, was entirely different from that of the pure solvent. The time necessary to saturate the catalyst was much prolonged, the hydrogenation of the carotene was considerably slower, and the catalyst remained highly dispersed in contrast to the speedy agglomeration which is always observed in the "clean solvent."

4).   The solvent used in this experiment was presaturated in the microhydrogenation apparatus and was scrupulously kept away from rubber and stop-cock grease.

In order to show that the lower values observed in the earlier experiments were not due to impurities or differences in the carotenes used at that time, a portion of one of the samples used previously (carotene from *Beta vulgaris*, L., var. *cicla*, L.; Carotene 3, Table I) was hydrogenated.   A value of 10.78 was observed for its hydrogenation ratio (Table I, Experiment 5).   The value obtained before was 9.70.

CONCLUSION

From these experiments it may be concluded that the hydrogenation ratio of both α- and β-carotene is 11 and that the discrepancies noted for *p*-menthane solutions were due to impurities in the solvent.

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THE SOURCE OF THE LIPIDS FOUND IN THE THORACIC DUCT LYMPH IN FASTING*

ENDOGENOUS LIPID SECRETION AND REABSORPTION IN THE BOWEL

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The presence of lipids, sometimes in considerable amounts, in the thoracic duct lymph of animals fasted 2 to 14 days has been observed by us in 60 dogs to date, and appears to be a constant feature of fasting. The lymph obtained during fasting is nearly always opalescent or milky in appearance, and contains from 250 to 1300 mg. of total fatty acids per 100 cc. The average value for the total fatty acids in the lymph of these fasting animals is 630 mg. per 100 cc., while 24 hours after a fat meal, the thoracic duct lymph is clear and contains usually not more than 200 mg. per 100 cc., showing a noteworthy increase in fasting. In six dogs, after a 48 hour fast, Eckstein (1) obtained clear lymph containing between 230 and 380 mg. of total fatty acids per 100 cc. The amount of lipids in the lymph depends only partly on the duration of the fast, the state of nutrition and the age of the animal being, among other factors, important (2). Our high lymph fat values during fasting were usually obtained in young dogs.

Some evidence was submitted in our earlier paper indicating that the source of these lipids during fasting is not the result of a *direct* passage of blood fat into the lymph spaces and lymph vessels, but is rather a part of the depot fat mobilized in fasting, the mechanism of the transport and the main local sources being as yet unknown. Further experiments have yielded information on these points.

* A preliminary report was made before the Illinois Branch of the Society for Experimental Biology and Medicine, March, 1933.

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EXPERIMENTAL

Young dogs weighing 7 to 12 kilos were used. The thoracic duct was cannulated under pentobarbital sodium (nembutal, Abbott), 30 mg. per kilo intravenously, and the anesthesia maintained by small additional injections whenever necessary. The lymph was analyzed for total fatty acids and cholesterol by Bloor's oxidation-titration method (3). By determining the lipid content of hourly samples and plotting the results, the normal lymph fat curve during fasting under anesthesia was obtained. The effect on this normal lymph fat curve of various known hormones, of certain drugs, and of other substances was studied during fasting. Such experiments with pilocarpine and secretin suggested the bowel as the main source of the lymph fat during fasting and, to

TABLE I

Normal Lymph Fat Curve during Fasting

Dog No.	Days fasted	Total fatty acids, mg per 100 cc.				
		Initial	2 hrs later	4 hrs later	5 hrs later	7 hrs. later
98	2	1357		443		318
1	3	914		471		
61	5	582			360	
58	7	424	388		333	

test this, in a number of animals thoracic duct lymph was collected after removal of the bowel. On two of these enterectomized dogs, the effect of pilocarpine on the lymph fat was studied. Finally, the effect of pilocarpine on the lymph fat of bile fistula dogs was observed.

Results

Normal Lymph Fat Curve during Fasting—The dogs were fasted 2 to 7 days. Lymph was collected continuously for 4 to 7 hours in hourly samples. The initial sample of lymph was opalescent or milky, as previously described, but in the succeeding hours became clear, or nearly clear. Table I shows the results obtained in four typical experiments.

Under these experimental conditions it is seen that the lipid content of the lymph decreases with time, most probably because of

factors connected with the narcosis. If the initial lipid value is only moderately high, the decline is less marked, since there is a minimal basic lipid content of the lymph.

Effect of Certain Known Hormones and of Other Substances on Normal Lymph Fat Curve following Fasting—After the initial lymph sample had been obtained following fasting, the change produced by the intravenous injection of certain drugs was observed. Separate dogs were used for each test. In this manner we examined the effects of insulin (two dogs), epinephrine (two dogs), thyroxine (two dogs), pituitrin (two dogs), glucose (three dogs), and a preparation of the anterior lobe of the hypophysis (two dogs), made according to the directions of Anselmino and Hoffmann (4). We observed no definite changes in the normal lymph fat curve under the above experimental conditions, the lipid content declining in 2 to 6 hours in a manner similar to the decline in the control animals.

Effect of Pilocarpine—1 mg. of pilocarpine per kilo of body weight was injected intravenously after the initial lymph sample had been collected. The injection of pilocarpine was followed immediately by marked salivation, marked contraction of the urinary bladder, and an *increased lymph flow*. (A little blood also appeared in the lymph probably due to the marked intestinal contraction caused by the pilocarpine.) Soon (10 to 20 minutes) the previous rate of flow was reestablished, and in the subsequent hours was similar to the rate observed in the control animals. The lymph was less opaque 2 to 3 hours after the pilocarpine injection, but gradually became milky so that at the 5th hour, the lipid content had increased, sometimes above the initial level. The data in five such experiments are shown in Table II.

It appears that in fasting dogs the intravenous injection of pilocarpine causes a marked increase in the lipid content of thoracic duct lymph, beginning 2 to 5 hours after the injection and lasting several hours.

Effect of Secretin—60 dog units of secretin (3.0 mg., vasodilatin-free) were injected intravenously in three equal portions in half hour intervals. (A dog unit of secretin is that amount which will increase the flow of pancreatic juice by 10 drops in 10 minutes in an average dog.) The injections were well tolerated and without any immediately noticeable effect. Subsequent decrease in the

milkiness of the lymph observed in the control animals was not
noticed in these animals; on the contrary, the lymph maintained
its initial opacity for 1 to 2 hours, became more milky 2 to 3 hours
after the injection, and less milky thereafter. The results are
given in Table III.

The intravenous injections of secretin cause a definite increase
in the lipid content of thoracic duct lymph of fasting dogs.

TABLE II

Lipid Content of Lymph during Fasting after Pilocarpine Injection

Dog No.	Days fasted	Total fatty acids, mg. per 100 cc.					
		Initial	2 hrs after injection	4 hrs after injection	5 hrs after injection	6 hrs after injection	8 hrs. after injection
62	3	886			690		1136
63	3	635		520		924	
56	6	731	535	914		665	
46	7	360		1385			
36	9	657	726	886			

TABLE III

Lipid Content of Lymph during Fasting after Secretin Injection

Dog No.	Days fasted	Total fatty acids, mg per 100 cc				
		Initial	1 hr after secretin	3 hrs after secretin	5 hrs after secretin	6 hrs after secretin
59	3	665		776		654
60	3	859		1173	886	
55	4	969	925	1660	985	

Effect of Removal of Bowel—Sub-total enterectomy was per-
formed aseptically on dogs under morphine-ether anesthesia:
the bowel was removed from the rectosigmoid junction to the mid-
portion of the duodenum, the free end of the duodenum being
brought to the outside through a stab wound, thus draining the
gastric juice, pancreatic juice, and bile to the outside. The ani-
mals were allowed to recover and were then fasted several days,
fluid (750 cc. of 0.9 per cent saline solution) being given twice
daily subcutaneously. The thoracic duct was then cannulated
under pentobarbital sodium anesthesia. The lymph obtained

from these animals was clear and occasionally yellowish. Dogs 44 and 51 in this series were given intravenous injections of pilocarpine, 1 mg. per kilo, after the initial samples of lymph had been

TABLE IV

Lipid Content of Lymph during Fasting in Enterectomized Dogs. Effect of Pilocarpine

Dog No.	Days fasted	Total fatty acids, mg. per 100 cc.	
		Initial	5 hrs. after pilocarpine
51*	3	248	220
44*	5	166	122
40	4	272	
53	6	313	
35	7	250	
42	8	165	

* Injected with pilocarpine after the initial lymph sample had been collected.

TABLE V

Lipid Content of Lymph during Fasting in Bile Fistula Dogs. Effect of Pilocarpine

Dog No	Days fasted	Total fatty acids, mg. per 100 cc			Initial cholesterol per 100 cc
		Initial	4 hrs after pilocarpine	6 hrs after pilocarpine	
					mg.
14*	4	386			158
18*	6	388			185
64†	4	1220		471	
65†	6	969		470	
49†	7	471	415		
50†	8	499	222		

* The gallbladder had been removed and the common bile duct cannulated aseptically before these dogs started fasting.

† The common bile duct had been cannulated after the initial lymph sample had been collected. Then pilocarpine was injected.

obtained. The collection of lymph was then continued for 6 hours. In both cases the lymph remained clear and the total fatty acid content was very low. The results of the experiments on the enterectomized animals are given in Table IV.

The thoracic duct lymph of fasted enterectomized animals has a low lipid content which is not increased by intravenous injections of pilocarpine.   The lymph resembles cervical lymph.

Effect of Bile Fistula—The gallbladder was removed and the common bile duct cannulated aseptically under morphine-ether anesthesia.   Attached to the cannula was a length of rubber tubing coiled in the abdomen, the free end of the tube emptying on the outside into a rubber bag held in place by an abdominal binder. The dogs were fasted 4 to 6 days after recovery, and then thoracic duct lymph obtained.   This lymph was clear and greenish yellow.   Table V shows that the lipid content is relatively low in spite of a high cholesterol content.   Evidently part of the fatty acids represents cholesterol esters in these lymphs.

Dogs were fasted 4 to 8 days, the thoracic duct cannulated under pentobarbital sodium anesthesia, and an initial sample of lymph collected for $\frac{1}{2}$ hour.   Then the common bile duct was cannulated, thus excluding bile from the intestine, and 1 mg. of pilocarpine per kilo injected intravenously, the collection of lymph being continued for several hours.   In these dogs, as is seen in Table V, pilocarpine fails to increase the lipid content of the lymph; on the contrary it continues to decline in the same way as in the normal fasting control dogs.

<div align="center">DISCUSSION</div>

Our finding that the thoracic duct lymph of fasted enterectomized dogs is clear and contains very little fat indicates clearly that the principal source of the milky lymph in fasting is the bowel. With the bowel removed, the thoracic duct lymph resembles cervical lymph in appearance and lipid content (2), which is apparently a constant minimal basic content in lymph.   The additional lipid content in the lymph of normal fasting dogs under anesthesia might come either (a) by direct passage of blood fat from the blood capillaries into the lymphatics, or (b) the fat might have been absorbed from the lumen of the bowel.   The first premise (a) is unlikely as we (2) and others (5) have shown that blood fat does not directly enter the lymphatics even when it leaves the blood stream rapidly and in large amounts.   Only the latter mechanism (b) is consistent with our findings; namely, that pilocarpine and secretin markedly increase the lipid content of lymph

during fasting, that when the bile is shunted from the intestine to the outside, the lipid content of the lymph is low and does not rise on the injection of pilocarpine, and that the lymph fat cannot be increased with pilocarpine in enterectomized animals.

In fasting the lipids in lymph coming from the lumen of the bowel might be reabsorbed bile fat. In dogs with the gallbladder removed, the fistula bile has been shown (6) to contain between 0.09 and 0.49 per cent of saponifiable material, the average being 0.314 per cent. The fact that in our experiments the presence of bile appeared to be necessary for the production of a milky lymph is consistent with this assumption. However, the presence of bile would be necessary in part at least for the absorption of fat from the lumen of the bowel regardless of the source of this fat. Furthermore, the amount of lipids contained in bile could not account for the amount of lipids found by us in lymph during fasting even assuming that all the bile fat is quantitatively reabsorbed; the 24 hour quantity of bile under normal nutritional conditions is 3.5 to 9.5 cc. per kilo (7), with an average of 6.5 cc. per kilo per 24 hours. On the basis of an average of 0.314 per cent saponifiable material, this bile is equivalent to 20 mg. of lipids per kilo per day, while we have found an average of 400 mg. per kilo per day in lymph during fasting. This discrepancy becomes even greater if we consider the fact that in fasting the bile production is greatly diminished.

If bile is not the source of the lipids in lymph during fasting but serves only to aid in its absorption, there remains only the following possibility: lipids are secreted from the blood by the mucous membrane of the bowel into the lumen where a part of the lipids undergoes reabsorption.

The idea of an excretion of lipids by the intestine is not new. In 1884 Müller (8) arrived at the conclusion that inasmuch as fatty material is present in large amounts in feces and meconium following fasting, it must represent some sort of an excretion. Hutchison (9) mentioned that the fairly constant percentage of fat in the feces of man and other animals suggests that the fecal fat has a function to perform, and that it is not a pure excretion. Extensive work in Bloor's laboratory by Hill and Bloor (10), Sperry and Bloor (11), Sperry (12), Angevine (13), and by others (14) has yielded quantitative results on this phenomenon. In

a summary of his work, Sperry (15) states that with dogs on a lipid-free diet an average fecal excretion of 219 ± 9.5 mg. of lipids per kilo per week was found.

In discussing the physiological importance of this endogenous lipid excretion Sperry suggests that the fecal lipids represent leakage of plasma lipids for the purpose of lubricating the intestine, and, in addition, for the purpose of removing undesirable or excess sterols from the organism.   This interpretation may be correct, but it assigns no physiological importance to the lipid reabsorption which, according to our findings, is far more extensive than the lipid excretion.   A comparison of the amounts of lipids found during fasting in lymph (assuming the 24 hour quantity of lymph during fasting to be one-sixteenth of the body weight) with the amounts found in feces indicates that about 90 per cent of all the fat secreted into the lumen of the bowel is reabsorbed.   If similar conditions prevail in man, then a 60 kilo man in fasting secretes daily between 13 and 50 gm. of lipids into the bowel, with a reabsorption of 11 to 48 gm. respectively, by the lymph system.   Actually there may be an even greater secretion of lipids into, and reabsorption from, the lumen of the bowel since part of the fat could be taken up by the blood vessels of the bowel as probably occurs with fed fat.

What possible purpose can the secretion and reabsorption of such relatively large amounts of lipids in fasting serve?

It might be that the reabsorbed fat serves as a vehicle for carrying undesirable sterols across the intestinal wall in solution (Sperry) and is then reabsorbed after it has fulfilled this mission. Under these circumstances one should expect to find a rather constant relationship between the amounts of the reabsorbed and excreted lipids.   This, however, is evidently not the case.   While the amount of the fecal lipids is remarkably constant, 219 ± 9.5 mg. per kilo per week, the lipid content of lymph during fasting is extremely variable, from 250 to 1300 mg. per 100 cc.

The following consideration appears to us to be more consistent with the available facts.   It has been found by a number of workers (quoted by Leathes and Raper (16)) that after fat feeding the chyle fat has a different melting point and iodine number than has the fat administered, indicating some modification by the intestinal mucosa during absorption.   Similar conclusions were arrived

at by Rony and Mortimer (17) on the basis of intravenous fat injection experiments.

Our finding of a relatively extensive lipid secretion and reabsorption in the bowel in fasting suggests that similarly some of the mobilized fat is acted upon by the intestinal mucosa before it can be utilized.

SUMMARY

1. Thoracic duct lymph of dogs fasted 2 to 14 days contains 0.25 to 1.3 per cent total fatty acids, with an average value for 60 dogs of 0.63 per cent.

2. The chief source of this fat is the bowel, according to the results of enterectomy experiments. The intestinal mucosa secretes fat into the lumen of the bowel in fasting. A portion of this fat is excreted with the feces and constitutes the endogenous lipid excretion; the major portion of the secreted lipids is hydrolyzed in the lumen of the bowel, reabsorbed into the intestinal mucosa, resynthesized there, and passed into the lacteals.

3. Pilocarpine and secretin increase the lipid content of thoracic duct lymph in fasting anesthetized (pentobarbital) dogs, after a latent period for pilocarpine of 2 to 5 hours. When bile is excluded from the intestine, pilocarpine does not cause an increase in the lipid content of the thoracic duct lymph, which is thought to be due to the absence of the favorable rôle that bile plays in the digestion and absorption of fat and not to the lipid content of bile *per se*.

4. It is suggested that the physiological importance of endogenous lipid reabsorption is to subject a part of the mobilized depot fat to certain modifications preliminary to utilization. In this way, the intestinal mucosa plays a rôle in intermediate fat metabolism.

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LABILE SULFUR IN PROTEINS[*]

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It has been shown (1) that cysteine, on heating with alkaline plumbite, yields all of its sulfur as lead sulfide. The quantitative nature of this reaction has been checked under conditions suitable for the estimation of small amounts of cystine.

5 cc. of a N hydrochloric acid solution containing 20 mg. of cystine were treated with 5 cc. of a 2 per cent solution of hydrated stannous chloride (containing just enough hydrochloric acid to furnish a clear solution) and gently boiled for 10 to 15 minutes to insure complete reduction to cysteine. To the hot solution there were then added 10 cc. of 10 per cent lead acetate solution, followed by 50 cc. of 25 per cent sodium hydroxide. The clear solution was boiled for 24 hours over a small flame under a reflux condenser.[1] At the end of this time all of the labile sulfur had separated in the form of lead sulfide. This was collected by centrifuge, washed twice with 1 per cent sodium hydroxide solution, and covered (in the centrifuge tube) with 0.5 gm. of potassium chlorate crystals. There were then added 4 to 5 cc. of a cold solution of 10 cc. of bromine in 150 cc. of concentrated hydrochloric acid diluted with 100 cc. of water. The whole was then gently stirred with a rod until the black color of the lead sulfide had disappeared, care being taken to avoid a rapid decomposition, which might permit the escape of undecomposed sulfur halides. At this point 1 to 2 cc. of concentrated hydrochloric acid were added; this caused the

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[1] The vapor in the flask effectively excludes air, which has been found to oxidize lead sulfide suspended in hot alkaline solutions.

decolorization of free bromine (by oxidation) and the liberation of chlorine. When all sulfur had dissolved, the mixture was gradually warmed on the water bath until evolution of chlorine ceased, then transferred to a small beaker, and evaporated to complete dryness. The residue was treated with two successive 20 cc. portions of hot 20 per cent hydrochloric acid, filtered to remove any silicic acid (derived from the decomposition flask), and again evaporated to dryness. The residue was dissolved in 50 cc. of 0.1 N HCl and sulfate precipitated with barium chloride. In eight such determinations, values falling within the limits of 97 to 107 per cent of the calculated amount were secured.

Similar experiments with purified proteins have indicated that the above procedure may be applied to the determination of "labile sulfur" either on hydrolysates or on the proteins direct, quantities of the latter being employed such that 2 to 6 mg. of sulfur were involved. In this case, the sample was first warmed for a few minutes with 50 cc. of water before adding the stannous chloride; the subsequent operations were exactly as described above.

From the results obtained with unhydrolyzed proteins (Table I) it will be seen that the labile sulfur values obtained with casein, cottonseed globulin, zein, and edestin are in reasonably good agreement with the cystine values secured by Jones, Gersdorff, and Moeller (2) for these proteins with the original method of Folin and Looney (3), and in the case of edestin by Sullivan and Hess (4), the methods of Sullivan and Okuda being employed. The labile sulfur values for casein, edestin, zein, and fibrin agree well with those obtained by the cuprous method for cystine recently reported by Vickery and White (5), but that for horse hemoglobin is definitely higher than the corresponding figure obtained by the latter workers. In the case of egg albumin, moreover, the labile sulfur value is considerably higher than the cystine-sulfur figure obtained by the Folin-Looney procedure, and somewhat greater than that observed by Sullivan.

In view of this discrepancy a few preliminary experiments have been made to ascertain whether egg white hydrolysates contain labile sulfur derivatives other than cystine or cysteine.

Indications were secured that cystine might, contrary to the general impression as to its behavior, be taken up from acid solu-

TABLE I

Labile Sulfur in Purified Proteins

Protein	Taken	Labile S	Cystine S from recorded observations				
			Folin-Looney (2)	Sullivan (4)	Okuda (4)	Gasometric (6)	Vickery-White (5)
	gm.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Casein*	5 00	0 106					
	5 00	0 098					
	5 00	0 106					0 122
"　†	3 00	0 087					
	3 00	0 080					
	5 00	0 082	0 069			0 25	0 06
Gelatin (Bacto)	10 0	0.021					
	10 0	0 019					
"　(Difco)	10 0	0 0214					
	10 0	0 0185					
	10 0	0 0227				0 00	
Cottonseed globulin†	1.50	0 283					
	1 50	0 275					
	1 50	0 281	0 28				
Zein from yellow corn†	1 50	0 203					
	1 50	0 214					
	1 50	0 221	0 22				0 242
Edestin†	1 00	0 361					
	1 00	0 353					
	1 00	0 345	0 26	0 31	0 34	0 46	0 333
Egg albumin	0 95	0 446	0 35‡				
	0 95	0 427					
	0 99	0 430					
	0 99	0 448	0 23	0 32	0 34	0 42	
Fibrin (beef)	1 00	0 450					
	1 00	0 468					
	1 00	0 455					0 403
Horse Hb (crystalline)	4 00	0 180					
	4 00	0 185					0 11

* Commercial sample "according to Hammarsten."

† Samples kindly furnished by Dr. H. B. Vickery.

‡ See reference (7).

tion by butyl alcohol to a small but appreciable extent. This point was tested experimentally by shaking 50 cc. quantities of solutions of cystine in n HCl and H_2SO_4 with three successive 200 cc. portions of butyl alcohol previously saturated with water. The

united alcoholic layers were distilled to dryness under reduced pressure; the residue was dissolved in 0.1 N HCl and made up to 100 cc. Aliquot portions were analyzed for total sulfur (8) and for cystine according to the procedures of Folin and Marenzi and of Sullivan (Table II).

TABLE II

Extraction of Cystine from Acid Solutions by Butyl Alcohol

Solvent	Cystine taken	Cystine found in		Method
		Alcohol	Acid	
	mg	*mg*	*mg*	
N HCl	120	12 5	107 5	Total S
" "	120	12 2	103.9	" "
" "	120	14 3	103 9	" "
" "	120	11 7	108 3	Folin-Marenzi
" "	120	14 5	105 5	"
" "	120	13 3	105 7	"
" "	120	0	109 6	Sullivan
" "	120	0	106 7	"
" "	120	0	108 3	"
" H_2SO_4	50	0	46 5	Folin-Marenzi
" "	50	0	48 0	"
" "	50	0	48 5	Sullivan
" "	50	0	49 0	"

TABLE III

Butyl Alcohol Extraction of Solutions of 100 Mg. of Cysteine in 100 Cc. of N HCl

Cysteine found				Method
Experiment I		Experiment II		
Alcohol	Acid	Alcohol	Acid	
mg.	*mg*	*mg*	*mg.*	
54.3	46 8	52 7	46 1	Total S
52 7	46 1			Labile "
28 0	29 4	20 5	35 3	Folin-Marenzi
29 9	25 8	22 2	34 2	Sullivan

A similar experiment (carried out in duplicate) with 100 mg. of cysteine in 100 cc. of N hydrochloric acid showed this substance to be much more readily extractable (Table III). In this case, however, the recovery, as indicated by both the Folin and the

Sullivan processes in which recrystallized cysteine hydrochloride was employed as standard, is far from complete. The analyses for total sulfur and for labile sulfur show satisfactory values, which indicate that about one-half of the cysteine had passed into the butyl alcohol.

The possibility that the apparent loss, or rather alteration, of cysteine during extraction might be due to the formation of butyl esters, peptides, or diketopiperazines was excluded by hydrolyzing portions of the resulting solutions, when no increase of color occurred with either colorimetric procedure. Nor can it be ascribed to aerial oxidation, for aeration of solutions of cysteine in N HCl in the cold for 24 hours had no effect in either test; when the aeration was carried out for 24 hours at the boiling temperature, losses of only about 10 per cent of developable color occurred in both the Folin-Marenzi and the Sullivan tests. The butyl alcohol solutions on evaporation left amorphous residues from which no individual substances were isolated. No explanation is offered for the effect, an investigation of which is in progress.

It is clear from the experiments summarized in Table II that cystine is extracted from N hydrochloric acid to a small, but appreciable, extent, and not at all from N sulfuric acid. It is also clear that the results of colorimetric determinations of cysteine can be interpreted only with considerable reserve in experiments involving extraction with butyl alcohol. In the case of cystine, this restriction applies to the highly specific Sullivan procedure only. The labile sulfur method, on the other hand, yields significant and reliable results.

Attempts were made to secure more detailed evidence of the nature of the sulfur in egg albumin, other than that existing in the form of cystine (or cysteine) and methionine (9). Dried egg white was investigated, rather than the crystalline albumin, owing to its more ready availability. Several grades of egg white were employed in the preliminary experiments. In no case was sulfate, whether inorganic or ethereal, present in detectable amounts.

The protein was hydrolyzed by boiling under a reflux with dilute hydrochloric or sulfuric acid until the biuret test was negative, in accordance with standard practise. When the acid was to be removed, this was done in various ways, as indicated in the footnote to Table IV. Decolorization with norit charcoal is necessary

when colorimetric procedures are to be applied; it is recognized that this practise invariably involves the removal of sulfur compounds by adsorption, but it could not be avoided.   The hydrolysate was diluted to standard volume and determinations of total sulfur, labile sulfur, and cystine by colorimetric methods were made on aliquot portions.

TABLE IV

Sulfur Distribution on Extracting Egg White Hydrolysates* with Butyl Alcohol

Experiment No.	Egg white	Hydrolysate				BuOH extraction	Ratio of cystine extracted to that in hydrolysate		Ratio of cystine in aqueous solution to that in hydrolysate	
		Total S per gm. egg white	$\frac{\text{Labile S}}{\text{Total S}}$	$\frac{\text{Cystine S}}{\text{Total S}}$			Folin-Marenzi method	Sullivan method	Folin-Marenzi method	Sullivan method
				Folin-Marenzi method	Sullivan method					
	grade	mg.								
1	I	8 5		0 176	0 093	Continuous, 182 hrs.	0 45	0	0 30	0 50
2	I	8 4		0 178	0 095	Continuous, 450 hrs.	0 53	0	0.30	0 55
3	II	10 4	0 44	0 280	0 280	Separatory funnel	0 15	0	0 95	0 75
4	III	16 6	0 37	0 34	0 33	" "	Trace	0	0 35	0 39

* *Experiments 1 and 2*—Hydrolyzed with H_2SO_4; decolorized with 1 gm. of norit per gm. of egg white; H_2SO_4 removed with $CaCO_3$ and finally with $Ba(OH)_2$.

Experiment 3—Hydrolyzed with HCl; decolorized with 0.5 gm. of norit per gm. of egg white; acid not removed.

Experiment 4—Hydrolyzed with HCl; free acid removed *in vacuo*; decolorized with 0.5 gm. of norit per gm. of egg white.

Extraction with butyl alcohol according to Dakin's method (10) was performed under reduced pressure in a simple continuous apparatus or by shaking in a separatory funnel with five successive portions of butyl alcohol equal in volume to the original aqueous solution.   The results of a few representative experiments are recorded in Table IV.   From these it can be inferred that the Folin-Marenzi "cystine sulfur," which with different samples varies from 18 to 35 per cent of the total sulfur in the decolorized

hydrolysates, is only slowly and incompletely extractable by butyl alcohol, while the cystine values, as determined by the Sullivan method, tend to disappear during extraction.

In a more elaborate experiment 1000 gm. of egg white (Mallinckrodt) were boiled gently over a free flame with a mixture of 2000 gm. of sulfuric acid and 6000 cc. of water until the biuret reaction had been negative for 4 hours, or about 40 hours in all. The humin was filtered off. It amounted to about 30 gm. and contained less than 0.5 per cent of the sulfur originally present. No hydrogen sulfide was evolved during the hydrolysis. The filtrate was freed of sulfate by the addition of excess of calcium carbonate, followed by exactly the necessary quantity of barium hydroxide, and decolorized by means of 437 gm. of norit.

The resulting solution, amounting to 19 liters, which was found to contain 7.98 gm. of sulfur and 87.53 gm. of nitrogen (0.82 and 9.0 per cent respectively of the weight of egg white originally taken), was extracted with butyl alcohol in a continuous apparatus under reduced pressure. The process was interrupted at intervals and the resulting extracts filtered. The fractions which had separated were all soluble in cold water. The butyl alcoholic filtrates were evaporated to dryness under reduced pressure; the earlier extractions yielded residues which could be subdivided into fractions respectively soluble and insoluble in cold water. Each fraction was analyzed for nitrogen, total sulfur, labile sulfur, and for cystine sulfur by the method of Folin and Marenzi.[2] In none was a positive response given in the Sullivan test for cystine. Qualitative tests were also carried out for thiolglyoxalines, Benedict's reagent for thiolhistidine being employed (11). Hunter's reaction for thiolglyoxalines (12) gave strictly parallel results when the fractions were not too deeply colored for its application.

From the results presented in Table V it appears that over 70 per cent of the total sulfur is extractable by butyl alcohol. Of this "extracted" sulfur, less than one-sixth exists in labile form, so

[2] The cystine sulfur values reported in Table V were determined after the fractions had stood in solution (under toluene) for 2 months in well stoppered containers. The freshly prepared solutions gave values varying from 50 to 100 per cent higher; this difference must have been due to the presence of spontaneously oxidizable contaminants capable of reducing the Folin-Marenzi reagent.

that the principal part of it is probably present in the form of methionine. After the experimental work was completed, it was shown by Pirie (13) that methionine is extractable by butyl alcohol from hydrochloric acid solution; the present evidence indicates

TABLE V

Butyl Alcohol Extraction of Hydrolysate from 1 Kilo of Egg White

Extract No	Fraction*	Total N	Total S	Labile S	Cystine S (Folin-Marenzi method)	Thiol-histidine (Benedict method)
		gm.	*gm*	*gm*	*gm.*	
1 (72 hrs.)	A	2 37	0 210	0 066	0 074	+++
	B	3 30	0 605	0 041	0 014	++
	C	0 143	0 115	0	0	−
2 (144 hrs.)	A	1 89	0 190	0 032	0 041	+++
	B	2 23	0 535	0 020	0 006	+
	C	0 200	0 105	0	0	−
3 (96 hrs.)	A	1 81	0 165	0 031	0 053	++++
	B	4 51	0 770	0 052	0 025	+++
	C	0 126	0 095	0	0	−
4 (180 hrs.)	A	2 85	0 190	0 039	0 050	++++
	B	6 10	1 010	0 058	0 037	++++
	C	0 130	0 070	0	0	−
5 (300 ")	A	1 62	0 210	0 031	0 041	++
	B	4 75	0 480	0 063	0 046	++++
	C	0 125	0 070	0	0	−
6 (300 ")	A	1 09	0 100	0 017	0 020	++
	B	4 90	0 160	0 094	0 067	+++
7 (240 ")	A	0 711	0 082	0 021	0 021	+
	B	4 03	0 223	0 099	0 075	−
8 (600 ")	A	0 688	0 043	0 010	0 010	−
	B	4 295	0 348	0 234	0 334	−
Total extracted. ...		47 87	5 776	0 908	0 914	
Aqueous residue .		33 46	1 594	1 052	0 222	
					0 235 (Sullivan method)	

* Fraction A, BuOH-soluble, H_2O-soluble; Fraction B, BuOH-insoluble, H_2O-soluble; Fraction C, BuOH-soluble, H_2O-insoluble.

that methionine can also be extracted from neutral solution, together with the other monoaminomonocarboxylic acids.

The failure to find cystine in the butyl alcohol extracts by the Sullivan reaction cannot be regarded as conclusive evidence of its

absence, owing to the observations, already noted, on the effect of butyl alcohol on this test. The less specific Folin-Marenzi procedure, on the other hand, gives a total value for cystine which closely agrees with that for labile sulfur. This may, however, be due merely to coincidence, since such good agreement was not observed in the individual fractions. The potential reducing power (Folin-Marenzi) had for some undetermined reason materially decreased after the solutions had been allowed to stand for 2 months in stoppered flasks.

Of the sulfur remaining in the aqueous solution after treatment with butyl alcohol, two-thirds exists in the labile form. The colorimetric tests for cystine, however, indicate the presence of only about one-fifth of the amount of cystine corresponding to this labile sulfur. Although extraction into butyl alcohol, as has been shown above, affects the magnitude of values obtained by the Sullivan procedure, this does not always appear to be the case with the Folin-Marenzi test. Indeed, the latter suffers from the disadvantage of non-specificity, often giving too high values on account of the presence of oxidizable substances devoid of sulfur (4). It therefore appears probable that the hydrolysate of egg white contains substances other than cystine which yield lead sulfide on heating with alkaline plumbite, and, like cystine, are not readily extractable by butyl alcohol.

Although it has been shown by Barger and Ewins (14) that ergothioneine does not give rise to sulfide on heating with alkali, the possibility must be considered that the closely related compound thiolhistidine may be present in the egg white hydrolysate and yield labile sulfur. The exploration of this has been reserved for the future.

The effect of extraction with butyl alcohol from hydrochloric acid solution is illustrated in the following experiment. 100 gm. of egg white (total S, 1.85 per cent; labile S, 0.65 per cent) were heated with 250 cc. of concentrated HCl on a steam bath for 40 hours. The biuret test was negative after 36 hours. After distilling off as much as possible of the free acid *in vacuo*, the residue was diluted with water and filtered. The humin precipitate contained 7.5 mg. of sulfur. The filtrate (still strongly acid to Congo red) was made up to 500 cc., and an aliquot portion was analyzed. A 100 cc. portion was shaken successively with 400,

300, 300, and 300 cc. quantities of butyl alcohol. The aqueous layer after this treatment, amounting to 58 cc., was made up to 100 cc. and analyzed. The combined butyl alcohol extracts were evaporated to dryness under reduced pressure and the residue dissolved in water, made up to 100 cc., and analyzed. The analytical results are presented in Table VI.

A similar experiment involved a partial fractionation of the amino acids extracted from an acid (HCl) hydrolysate by means of butyl alcohol. A mixture of 2 kilos of egg white (the same material as was employed in the preceding experiment) and 6 liters of concentrated hydrochloric acid was heated on the steam bath under a reflux for 35 hours. The humin was removed and washed. Filtrate and washings (8 liters) were evaporated to

TABLE VI

Sulfur Distribution in Hydrolysate from 100 Gm. of Egg White on Extraction from HCl Solution with Butyl Alcohol

	Nitro-gen	Total S	Labile S	Cystine S	
				Folin-Marenzi method	Sullivan method
	gm.	*gm.*	*ym.*	*gm*	*gm*
Hydrolysate..	10 8	1 66	0 62	0 55	0 55
Butyl alcohol solution	6 64	0 94	0 15	0	0
Aqueous solution . . .	4 35	0 63	0 46	0 21	0 215
Per cent recovery on extraction .	102	95	98	35	39

dryness *in vacuo*. The residue was twice redissolved in 3 liters of water and again evaporated, after which 1075 cc. of sulfuric acid, diluted with 2 liters of water, were added and the evaporation continued for 40 hours, with continual addition of water to maintain constant volume. The sulfuric acid was then removed by addition of the calculated amount (1915 gm.) of calcium carbonate, followed by exactly the necessary quantity of barium hydroxide. The resulting solution, which was acid to litmus but not to Congo red, was diluted to 8 liters and a 200 cc. portion removed for analysis.

The remaining 7800 cc., corresponding to 1945 gm. of egg white, were extracted in lots of 750 cc. with three successive 750 cc. portions of butyl alcohol; after the last extraction, the aqueous

phases had contracted from 750 to 490 cc. The combined butyl alcohol solutions were concentrated under reduced pressure to 2900 cc., and a yellow, amorphous precipitate (Fraction A) was removed. This was dissolved in 1500 cc. of concentrated hydrochloric acid and the solution boiled for 4 hours to hydrolyze any esters and anhydrides, and diluted to 2000 cc. Analysis of aliquot samples showed the presence of 20.66 gm. of nitrogen, 2.76 gm. of total sulfur, 0.88 gm. of labile sulfur, and 0.202 gm. of cystine sulfur (Folin-Marenzi). The Sullivan test was negative. The butyl alcoholic filtrate was evaporated to dryness, and the brown, gummy residue (Fraction B) boiled for 4 hours with 1 liter of concentrated hydrochloric acid and analyzed.

<div align="center">

TABLE VII

Sulfur Distribution in Fractions of Egg White Hydrolysate Extracted from HCl Solution by Butyl Alcohol*

</div>

	Nitrogen	Total S	Labile S	Cystine S Folin-Marenzi method	Cystine S Sullivan method
Original hydrolysate	111 5	10 4	4 6	2 9	2 9
BuOH Extract A	10 6	1 42	0 45	0 104	0
" " B	5 3	0 40	0	0 073	0
" " C	22 0	2 86	0 80	0 274	0
Aqueous solution	73 4	5 96	3 39	2 74	2 19
Total	111 3	10 64	4 64	3 19	2 19

* Figures show components in mg. per gm. of egg white.

As the analyses indicated insufficient extraction, the aqueous residue (5 liters) was concentrated to 4 liters, strongly acidified to Congo red with hydrochloric acid, and extracted in 750 cc. lots, each with two successive 750 cc. portions of butyl alcohol. The united butyl alcoholic solution was evaporated to dryness under reduced pressure; the residue (Fraction C) was boiled as above with hydrochloric acid, diluted to 2 liters, and analyzed. The residual aqueous solution was also analyzed.

A summary of all of the analyses, in which the results (corrected for withdrawal of aliquot samples) are expressed as mg. of each component per gm. of egg white, is presented in Table VII. These

figures indicate that about one-quarter of the labile sulfur was extracted by the butyl alcohol.   That remaining in the aqueous solution was largely in the form of cystine, as shown by the approximation of the labile sulfur to the Sullivan value; that extracted consisted only to a small extent of cystine, for the total of the Folin-Marenzi values amounted to little more than one-third of the total labile sulfur values.   Moreover, this extracted cystine had undergone the change which rendered the Sullivan test negative.   These results again point strongly to the presence in egg white hydrolysate of substances which contain labile sulfur but are not cystine.

The probability that the non-labile sulfur compounds consist largely of methionine gains strength from the recent report by Pirie (15) that a 2.5 per cent yield of this amino acid is obtainable from egg albumin.   This yield corresponds to a methionine-sulfur content of 0.54 per cent, a value which corresponds very closely to the difference between the figures for total sulfur and labile sulfur as determined in the above experiment.

An attempt was made to separate the components of the united butyl alcohol-soluble fractions by benzoylation and recrystallization from carbon tetrachloride.   The bulk of the sulfur-containing components collected in the final oily mother liquor.   This was dissolved in N sodium hydroxide and acetic acid added until no further precipitation occurred; the resulting oil was treated with 500 cc. of acetone and 3.5 liters of ether, when it separated into two layers.   The lower layer contained 25 per cent of the sulfur originally present, and of this one-half was in the labile form.   As no crystalline products were secured, this method of attack was abandoned.

As has been pointed out, in the various experiments involving extraction with butyl alcohol no loss of labile sulfur occurred, but the cystine taken up by the alcohol was apparently irreversibly altered in such a way that it failed to respond to the Sullivan test. With the idea of evading this difficulty, experiments were undertaken in which butyl alcohol extraction was avoided.   After hydrolysis with hydrochloric acid, the resulting amino acids were benzoylated, and an attempt was made to separate the resulting products.

The hydrolysate from 97 gm. of egg white was evaporated as

completely as possible under reduced pressure; the residue was dissolved in water and treated with a 50 per cent excess (based on the total nitrogen) of benzoyl chloride and sufficient sodium hydroxide and bicarbonate to maintain neutrality. After adding excess of acid and chilling, the semisolid product was washed with water and dissolved in a small amount of acetone.

The aqueous phase was concentrated under reduced pressure to a small volume, when an oil separated, together with sodium

TABLE VIII

Analytical Returns* on Fractions (from Flow Sheet) of Benzoylamino Acids

Fraction No	Nitrogen	Total S	Labile S / Total S	Cystine S / Total S	
				Folin-Marenzi method	Sullivan method
	per cent	per cent			
1	3 7	6 4	0	0	0
2	9 3	0			
3	3 5	4 7	0 27	0 07	0
4	6 1	Trace			
5	14 5	18 2	0 36	0 37	0 42
6	13 5	12 5	0 33	0 24	0 23
7	0	0			
8	0	0			
9	0 9	2 1	0	Trace	0
10	10 2	14 6	0 18	0 11	0
11	4 0	0			
12	25 9	32 7	0 29	0 21	0
Total ..	91 6	91 2			

* Percentages are referred to corresponding values in the original hydrolysate.

chloride. This oil was taken up in ether; it contained no sulfur. The water-soluble portion, however, contained sulfur amounting to 6.4 per cent of the total.

The acetone solution of the benzoylamino acids was diluted with acetone to 12 liters, when 5.07 gm. of an amorphous, brown precipitate separated (Fraction 3).

Subsequent fractions were secured according to the scheme indicated in the flow sheet. The analytical figures on these are recorded in Table VIII.

While in none of the fractions was more than one-third of the sulfur present in labile form, the maximum concentration occurred

*Separation of Benzoyl Derivatives**

Crude benzoylamino acids from 97 gm. egg white

```
            ┌──────────────────────────┴──────────────────────────┐
    Aqueous solution, con-                           Acetone-soluble, diluted
        centrated                                       to 12 l. with acetone
    ┌───────────┴───────────┐              ┌──────────────┴──────────────┐
Ether-soluble    Crystals (1)      Precipitate (3)      Filtrate, con-
  oil (2)                                                  centrated
                                          ┌──────────────────┴──────────────────┐
                                      Crystals (4)        Mother liquor, concentrated
                                                            and toluene added
                                               ┌──────────────────┴──────────────────┐
                                        Toluene-insoluble        Toluene solution, con-
                                             oil (5)                  centrated
                                                        ┌──────────────┴──────────────┐
                                                  Crystals (6)      Filtrate, 1 wk. at 0·5°
                                                             ┌──────────────┴──────────────┐
                                                       Crystals (7)      Filtrate, 3 wks. at 0·5°
                                                          ┌──────────────┴──────────────┐
                                                   Filtrate (2 layers)           Crystals (8)
                                              ┌──────────┴──────────┐
                                      Lower layer, extracted     Upper layer, concen-
                                       with alcohol-ether             trated
                                      ┌──────────┴──────────┐      ┌──────────┴──────────┐
                                  Insoluble (11)  Ether-soluble  Crystals (9)   Filtrate (10)
                                                    oil (12)
```

*The figures in parentheses designate the fractions referred to in Table VIII.

in the fractions which were most readily soluble in acetone and insoluble or sparingly soluble in toluene (Fractions 5 and 6).

In these fractions the labile sulfur appears to exist largely in the form of cystine.

On the other hand, the acetone-insoluble fraction (Fraction 3) contains 27 per cent of its sulfur in the labile form, but none of this labile sulfur can be accounted for as cystine, by either of the colorimetric tests (performed after suitable acid hydrolysis).

The final fractions (Fractions 10 and 12) most readily soluble in ether and toluene also show fairly high $S:N$ and labile $S:$total S ratios. The labile sulfur in these cases must exist largely in the disulfide (or sulfhydryl) form, since the Folin-Marenzi values are high, but cannot be present as cystine as the Sullivan reaction is negative.

DISCUSSION

The reproducibility of the values for labile sulfur obtainable with purified proteins entitles the method to be regarded as capable of furnishing one more analytical characteristic. It appears probable that in many proteins this value will approximate that for cystine sulfur; the case of egg albumin, however, and possibly of others, points to the possible presence in proteins of components other than cystine or cysteine which yield lead sulfide on heating with alkaline plumbite.

This view is supported by the preliminary observations here reported. On prolonged extraction of a neutral hydrolysate of egg white with butyl alcohol, over 70 per cent of the total sulfur passes into the alcohol. Of this sulfur about 15 per cent is in a labile form; the balance is undoubtedly mainly present as methionine, but appears to include some thiolglyoxaline. The labile sulfur in the butyl alcohol extract appears to exist substantially in the disulfide or sulfhydryl condition, as is shown by the close agreement of the values for labile sulfur and cystine sulfur as determined by the method of Folin and Marenzi. What proportion of it is ascribable to cystine was not determined, owing to an unexplained alteration of cystine, caused by extraction with butyl alcohol, which inhibits the more specific reaction of Sullivan. It is planned to investigate this point with the aid of the cuprous precipitation method recently described by Vickery and White.

The neutral aqueous solution remaining after the extraction contains 22 per cent of the sulfur originally present. About two-

thirds of this exists in labile form, but concordant determinations by the methods of Folin and Marenzi and of Sullivan indicate that only about a quarter of the labile sulfur is present as disulfide, all of which is in the form of cystine. The major part of the non-extracted labile sulfur, amounting to one-tenth of the total sulfur in the hydrolysate, thus exists in some unrecognized form.

The results of experiments involving extraction of hydrolysates containing free hydrochloric acid lead to similar conclusions.

Of particular interest is the acetone-insoluble fraction of the benzoylated hydrolysate of egg white. While this contained only 4.7 per cent of the original total sulfur, one-quarter of its sulfur was of a labile variety which gave no response to the test of Folin and Marenzi, and therefore cannot have existed in the disulfide or sulfhydryl condition. The fraction in question differed markedly from those containing benzoylcystine, which is readily soluble in acetone.

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ACETONE DERIVATIVES OF *d*-RIBOSE

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The acetone derivatives of glucose have served as the key
substances for the preparation of various partially substituted
derivatives of known structure. In the case of those hexoses whose
acetone derivatives did not possess a suitable structure—mannose
and galactose—but few monosubstituted derivatives of known
structure have been prepared. In this laboratory the acetone
derivatives of glucose served as starting material for the prepara-
tion of partially phosphorylated derivatives.[1] In connection with
the work on nucleic acids it was desired to prepare *d*-ribose-3-
phosphoric acid and *d*-ribose-5-phosphoric acid. The monoace-
tone derivative of ribose was envisaged as a good starting material
for this purpose. The number and the structure of the mono-
substituted derivatives obtainable from it naturally will depend
upon the structure of the acetone ribose.

Monoacetone-d-Ribose—From the very start it was observed
that the condensation of ribose with acetone proceeded in a pecul-
iar way. In the case of other monoses the diacetone derivative
is the predominating product of the reaction. In the case of
ribose, however, the diacetone derivative was not detected, but by
the usual treatment with pure acetone in the presence of 0.2 per
cent sulfuric acid and anhydrous copper sulfate two condensation
products were obtained which were separated by fractional dis-
tillation. The lower boiling fraction, which was obtained in about
15 per cent yield, crystallized in the receiving flask; the high boiling
fraction (about 75 per cent) thus far has not been crystallized,

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* Commonwealth Fund Fellow.
[1] Raymond, A. L., and Levene, P. A., *J. Biol. Chem.*, **83**, 619 (1929).
Levene, P. A., and Raymond, A. L., *J. Biol. Chem.*, **89**, 479 (1930).

and the remainder was not distilled. The *syrupy* distillate consisted in the main of isopropylidene ribofuranose of the structure (I).

For the purpose of purification of the isopropylidene-*d*-ribofuranose, the crude material was acetylated, the diacetyl isopropylideneribose was distilled, and the distillate deacetylated. This product had the elementary composition required by theory for a monoacetone ribose. There remained to be established, the position of the acetone residue and the ring structure of the substance.

In its reducing power the monoacetone ribose resembled monoacetone mannose.[2] It did not reduce Fehling's solution, but on the other hand was oxidized by alkaline hypoiodite solution. Thus it is evident that carbon atom (1) is not involved in the condensation with acetone.

Ring Structure of Monoacetone Ribose—The allocation of the isopropylidene group is not a simple matter but depends upon a knowledge of its ring structure. *A priori*, the pyranose and the furanose ring structures are equally possible. The ribopyranose permits equally well the formation of either a 2,3- or a 3,4-monoacetone derivative, whereas the furanose is most likely to form a 2,3 derivative. The following experimental evidence points toward a furanose structure for monoacetone ribose.

It readily forms a methyl monoacetone methylriboside (II) on methylation by the Purdie method. The structure of this dimethyl derivative was recognized by its behavior on heating in methyl alcohol containing dry hydrogen chloride, yielding under these conditions not a dimethyl methylriboside but a dimethyl derivative which, on hydrolysis with aqueous mineral acid, led to a monomethylribose (III) which formed a monomethylribo-*p*-bromophenylosazone. The rate of glycoside formation of the monomethyl sugar indicated a furanoside formation only, thus permitting the allocation of the methyl group in position (5).

On exhaustive methylation with subsequent hydrolysis, the monomethyl methylriboside obtained from the monoacetone derivative formed a trimethylribose (V) which in its physical properties is identical with the trimethyl-*d*-ribofuranose previously

[2] Freudenberg, K., Dürr, W., and von Hochstetter, H., *Ber. chem. Ges.*, **61**, 1735 (1928).

described by Levene and Tipson.[3]   Furthermore, the trimethylribose on oxidation with bromine formed the trimethyl-γ-ribonolactone[4] (VI).

Peculiar Behavior of Monoacetone Ribose towards p-Toluenesulfonyl Chloride—By treatment with 1 equivalent of the reagent, under the conditions generally leading to a mono-*p*-toluenesulfonyl substitution product, the monoacetone ribose forms a di-*p*-toluenesulfonyl derivative (VII).   One of these groups is readily substituted by iodine, a reaction introduced by Oldham and Rutherford[5] for identification of primary alcoholic groups.   Indeed, the iodine could not be easily removed by warming the substance for a short time with a dilute aqueous solution of sodium hydroxide, nor by shaking with an aqueous solution of silver nitrate.   Thus it would seem that the second *p*-toluenesulfonyl group is located in position (1), unless the acetone residue migrated during the course of the reaction.   The structure of the di-*p*-toluenesulfonyl derivative will be further investigated. It may here be added that the iodo derivative (VIII) is readily converted into the mononitrate (IX).

All these observations warrant the assumption that the hydroxyl in position (5) of monoacetone ribose is unsubstituted and hence the furanose structure may be assigned to it.

Allocation of Isopropylidene Radicle—The furanose structure of the monoacetone ribose, its free reducing group, and the fact that the hydroxyl in position (5) is unsubstituted leave carbon atoms (2) and (3) as the only possible positions of the isopropylidene radicle.   Thus, the detailed structure of the monoacetone ribose as 2,3-isopropylidene ribofuranose is expressed by formula (I).

The question naturally arises as to the causes which bring about the exclusive formation of a furanose structure when the hydroxyl in position (5) remains unsubstituted.   The answer is found in the three dimensional model of the substance, which shows that for a 2,3-monoacetone ribose the 5-membered ring is the more stable. It may be mentioned that the position of the acetone residue on carbon atoms (2) and (3) explains the failure of ribose to form a

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[3] Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **97**, 491 (1932).

[4] Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **94**, 809 (1931-32).

[5] Oldham, J. W. H., and Rutherford, J. K., *J. Am. Chem. Soc.*, **54**, 366 (1932).

diacetone derivative. It is also noteworthy that in the case of rhamnose, in which the hydroxyls of carbon atoms (2) and (3) are also in the *cis* position, Fischer[6] describes only a monoacetone derivative. It is planned to investigate in this laboratory the structure of this derivative as well as the acetone derivatives of lyxose and talose.

Monoacetone Anhydroriboses—The low boiling, crystalline fraction referred to earlier had the elementary composition corresponding to a monoacetone anhydroribose. As was to be expected with a substance without free hydroxyl groups, it failed to be acetylated and *p*-toluenesulfonated. It was not oxidized by sodium hypoiodite and did not reduce Fehling's solution until hydrolyzed by dilute mineral acid in the cold. Treatment of an alcoholic solution with sodium methylate at 125° failed to open the anhydro ring. Bearing in mind the position of the acetone group in the monoacetone ribose, it may be assumed that the substance had the structure of monoacetone-1,5-anhydroribofuranose (X).

A second crystalline compound was obtained in one condensation which was performed in the presence of hydrogen chloride in place of sulfuric acid. This compound had the same composition as the one mentioned above but its melting point was 30° higher. As the substance was available in only very small quantity, a detailed description of it is postponed until a later date.

Further work on the two anhydro derivatives as well as on the substitution derivatives of the isopropylidene-*d*-ribofuranose is in progress.

<div align="center">EXPERIMENTAL</div>

Condensation of d-Ribose with Acetone—The quality of the acetone used in the condensation to be described was found to be important. Even should the acetone contain only a very small quantity of methyl alcohol, the ribose condenses with this to give a monoacetone methylriboside. The acetone used in the following condensations was prepared from its bisulfite compound.

A typical condensation was carried out as follows: 20 gm. of dry, finely powdered crystalline ribose were suspended in 400 cc. of acetone containing 0.2 per cent of sulfuric acid. 40 gm. of

[6] Fischer, E., *Ber. chem. Ges.*, **28**, 1162 (1895).

$$\text{I} \rightarrow \text{II} \rightarrow \text{III} \rightarrow \text{IV} \rightarrow \text{V} \rightarrow \text{VI}$$

I
```
 ┌──────────────┐
 CH·OH
H·C·O      CH₃
        C        O
H·C·O      CH₃
H·C
 └──────────────┘
 CH₂OH
```

II
```
 ┌──────────────┐
 CH·OMe
H·C·O      CH₃
        C        O
H·C·O      CH₃
H·C
 └──────────────┘
 CH₂OMe
```

III
```
 ┌──────────────┐
 CH·OH
H·C·OH          O
H·C·OH
H·C
 └──────────────┘
 CH₂OMe
```

IV
```
 CH·OMe
H·C·OMe          O
H·C·OMe
H·C
 CH₂OMe
```

V
```
 CH·OH
H·C·OMe          O
H·C·OMe
H·C
 CH₂OMe
```

VI
```
 CO
H·C·OMe          O
H·C·OMe
H·C
 CH₂OMe
```

VII
```
 CH·OSO₂——⟨  ⟩——CH₃
H·C·O      CH₃
        C          O
H·C·O      CH₃
H·C
 CH₂·OSO₂——⟨  ⟩——CH₃
```

VIII
```
 CH·OSO₂——⟨  ⟩—CH₃
H·C·O      CH₃
        C          O
H·C·O      CH₃
H·C
 CH₂·I
```

IX
```
 CH·OSO₂——⟨  ⟩—CH₃
H·C·O      CH₃
        C          O
H·C·O      CH₃
H·C
 CH₂·ONO₂
```

X
```
 H·C
H·C·O      CH₃
        C          O
H·C·O      CH₃
H·C
 CH₂
```

191
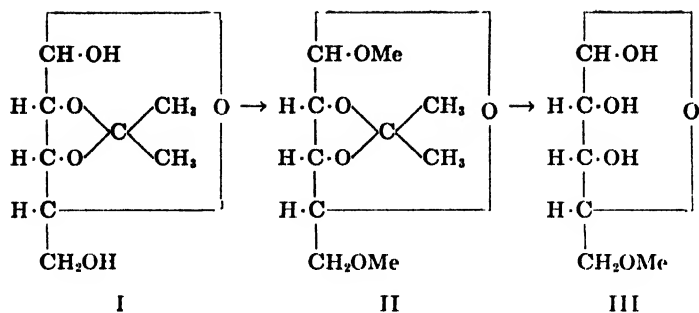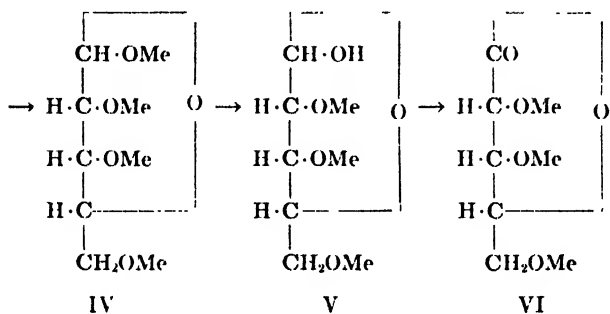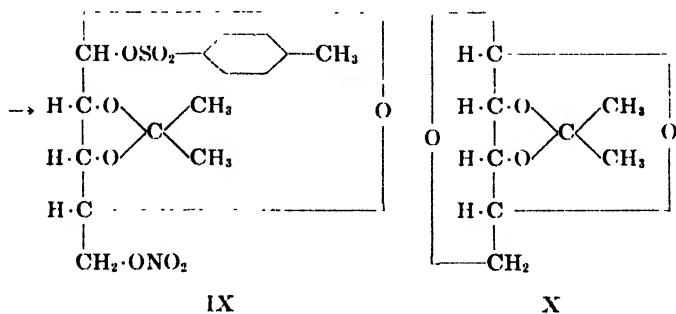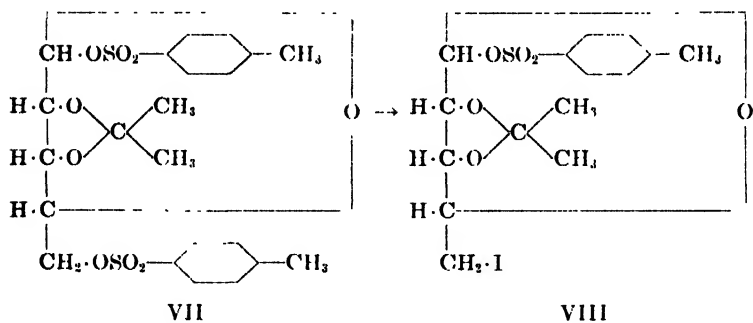
anhydrous copper sulfate were added and the mixture was shaken at 37° for 20 hours, after which the supernatant liquor no longer reduced boiling Fehling's solution. The copper sulfate was filtered off, washed thoroughly with small quantities of acetone, and the filtrate rendered neutral by shaking with calcium hydroxide for 1 hour. The calcium sulfate and excess calcium hydroxide were removed by filtration and thoroughly washed with small quantities of acetone. The combined filtrate and washings were then evaporated to dryness under reduced pressure at a temperature not exceeding 40°. Yield, 18 to 21 gm. of pale yellow, viscous syrup.

The product was fractionally distilled under reduced pressure and the following fractions were obtained: (1) 2.5 to 3.0 gm. of a colorless syrupy product which crystallized immediately in the receiver in long white needles; the boiling point was 55–60° at 0.05 mm. After recrystallization from methyl alcohol, the melting point was 61–62°. (2) 13.5 to 14.8 gm. of a pale yellow, viscous syrup; the boiling point was 110–117° at 0.05 mm.

Isolation of Monoacetone Ribofuranose from Fraction 2

Acetylation—Purification of 11.2 gm. of the main fraction was carried out by acetylation with acetic anhydride (20 cc.) in pyridine solution (27 cc.). The mixture was allowed to stand at 0° overnight and then poured onto ice with vigorous stirring. The ice-cold aqueous solution was extracted repeatedly with small quantities of chloroform and the combined chloroform extracts were washed with ice-cold dilute sulfuric acid, ice-cold sodium bicarbonate solution, and finally with ice-cold water. The chloroform solution was then dried over anhydrous sodium sulfate and the solvent removed under diminished pressure, giving a pale yellow syrup. Yield 94 per cent.

The product was fractionally distilled under reduced pressure and a main fraction (80 per cent) was obtained which distilled at 119–121° at 0.1 mm. It was a colorless, mobile syrup having $n_D^{29} = 1.4476$. The substance had the following composition.

5.480 mg. substance: 10 655 mg. CO_2 and 3.395 mg. H_2O
101.1 "   "   required 7.45 cc. 0.1 N NaOH
$C_5H_{12}O_5 \cdot (OCCH_3)_2$. Calculated. C 52.5, H 6.6, $OCCH_3$ 31.4
                              Found.   " 53.1, " 6.9,   "   31.7

Deacetylation of Diacetyl Monoacetone Ribose—Deacetylation of the diacetyl monoacetone ribose (14.1 gm.) was carried out by dissolving the substance in methyl alcohol (300 cc.) and adding to the solution at 0°, one-thirtieth of the theoretical amount of barium methylate,[7] which was then allowed to stand at 0° overnight. The solution was now saturated with carbon dioxide, 60 cc. of water added, and the solution again saturated with carbon dioxide. After the addition of a little charcoal, the solution was boiled for a few minutes, filtered, and the solvent removed by evaporation under reduced pressure. The final traces of water were removed by distilling three times with small quantities of benzene and three times with absolute alcohol. A colorless, viscous syrup was obtained. Yield 90 per cent. $n_D^{20} = 1.4642$. The substance had the following composition.

4.856 mg. substance: 9.065 mg. CO_2 and 3.220 mg. H_2O.
$$C_8H_{14}O_6.$$ Calculated. C 50.5, H 7.4
Found. " 50.9, " 7.4
46.9 mg. substance required 3.59 cc. 0.1 N sodium thiosulfate. Reducing power = 72.8.[8]

Preparation of Methyl Monoacetone Methylriboside—The monoacetone ribose (12 gm.) was methylated by means of Purdie's reagents (MeI, 60 cc.; and Ag_2O, 68 gm.) and the product isolated as a colorless syrup. The methylation was repeated using half the above quantities of methylating agents and the product was isolated as a colorless, mobile syrup which distilled at 62–65° at 0.03 mm. pressure, and had $n_D^{23} = 1.4383$. Yield 10.1 gm. The substance had the following composition.

5.575 mg. substance: 11.175 mg. CO_2 and 4.080 mg. H_2O
8.996 "   "   : 18.680 " AgI
$$C_5H_{12}O_4 (OCH_3)_2.$$ Calculated. C 55.2, H 8.3, OCH_3 28.5
Found. " 54.5, " 8.2, " 27.4

Treatment of Methyl Monoacetone Methylriboside with Methyl Alcoholic Hydrogen Chloride—Methyl monoacetone methylriboside (7.0 gm.) was dissolved in methyl alcohol containing 0.4 gm. of dry hydrogen chloride and heated for 23 hours at 70° in a sealed tube.

[7] Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **93**, 631 (1931).

[8] Levene, P. A., Raymond, A. L., and Dillon, R. T., *J. Biol. Chem.*, **95**, 699 (1932).

The hydrogen chloride was then neutralized with silver carbonate, the silver salts filtered off, and the methyl alcohol removed by evaporation under diminished pressure.   Yield 4.5 gm.

The product was fractionated under high vacuum, the main fraction being unchanged methyl monoacetone methylriboside, and a small second fraction (20 per cent) was obtained boiling at 70–77° at 0.12 mm. which was essentially monomethyl methylriboside.   It had the following methoxyl content.

6 295 mg. substance: 16 300 mg. AgI
$C_6H_8O_3$ $(OCH_3)_2$. Calculated, OCH_3 34.8; found, OCH_3 34.2

Preparation of Monomethyl Ribofuranose—The methyl monoacetone methylriboside (9.7 gm.) was hydrolyzed with 0.04 N hydrochloric acid (120 cc.) at 100° until the rotation became constant (in 120 minutes; $[\alpha]_D^{26} = +26.97°$). The solution was rendered neutral with barium carbonate, filtered, and the water removed under diminished pressure, at a temperature not exceeding 40°. The dry residue was repeatedly extracted with chloroform, the extract yielding, on removal of the chloroform, 7.5 gm. of a pale yellow, viscous syrup which reduced boiling Fehling's solution. The substance had the following composition.

4.420 mg. substance: 7 105 mg. CO_2 and 2 960 mg. H_2O
4 878 "   "   : 7 085 "   AgI
$C_6H_{12}O_5$. Calculated. C 43.9, H 7 3, OCH_3 18 8
          Found.   " 43 8, " 7 5, "   19 2

The change in rotation of a 0.0344 M solution of the sugar (corrected for ash) in methyl alcohol containing 0.5 per cent of dry hydrogen chloride was measured at room temperature and at 76° ($l = 2$ dm.).   See Table I.

Preparation of p-Bromophenylosazone of Monomethyl Ribose—The *p*-bromophenylosazone was prepared in the usual way and was isolated as long yellow needles. The melting point was 161–162° with darkening.   It had the following composition.

5 100 mg. substance: 0.519 cc. N_2 (23° at 755 mm.)
6 323 "   "   : 3.122 mg. AgI
$C_{18}H_{19}O_3N_4Br_2$. Calculated. N 11.2, OCH_3 6.2
                  Found.   " 11.6, "   6 5

The substance had the following specific rotation.

$$[\alpha]_D^{26} = \frac{-0.24° \times 100}{0.5 \times 1.00} = -48.0° \text{ (in absolute alcohol-pyridine, 3:2)}$$

Preparation of Monomethyl Methylriboside--The methylriboside was prepared by dissolving the monomethyl ribose (7.5 gm.) in 80 cc. of methyl alcohol containing 0.5 per cent of dry hydrogen chloride and allowing the solution to stand at room temperature. After 20 hours, since the solution no longer reduced boiling Fehling's solution, the acid was neutralized with silver carbonate.

TABLE I*

Glycoside Formation of 5-Methyl Ribose

At room temperature			At 76°	
Time	$[\alpha]_D^{20}$		Time	$[\alpha]_D^{26}$
min	*degrees*		*hrs*	*degrees*
2 5	-13 1		2	-5 2
4	-14 3		20	-2 6
6	-10 9			
10	-10 0			
20	-5 7			
30	-4 8			
75	-3 0			
140	-1 8			
300	-1 8			
1440	-2 4			

* We are indebted to Dr. A. L. Raymond for carrying out this experiment.

After removal of the silver salts by filtration, the methyl alcohol was removed under diminished pressure leaving a fairly mobile syrup.   Yield 7.5 gm.

Preparation of Trimethyl Methylriboside—The methyl methylriboside was methylated twice with Purdie's reagents giving a pale yellow, mobile syrup.   Yield 7.8 gm.   On distillation under high vacuum, 6.5 gm. of colorless, mobile syrup were obtained (b.p. 68° at 0.05 mm.), having $n_D^{29} = 1.4369$.   The substance had the following composition.

4.680 mg. substance: 21 070 mg. AgI

$C_5H_6O \cdot (OCH_3)_4$.   Calculated, OCH_3 60 2; found, OCH_3 59 5

Hydrolysis of Trimethyl Methyriboside—1.6046 gm. of trimethyl methylriboside were dissolved in 150 cc. of N/15 hydrochloric acid and heated at 75°. After various intervals of time, the solution was cooled to room temperature and the following polarimetric readings (recorded in Table II) were obtained.

The proportion of reducing sugar in the solution was finally estimated by a Willstätter titration[9] which showed there was 102.7 per cent after 60 minutes.

The solution was now rendered neutral with barium carbonate, and after removal of the barium salts by filtration, the water was removed under diminished pressure, the residue being dried by repeated distillation with benzene and absolute alcohol. The dry residue was extracted repeatedly with chloroform and after re-

TABLE II

Hydrolysis of Trimethyl Methylriboside

Time	$[\alpha]_D^{25}$
min	*degrees*
0	+13 0
30	+19 5
60	+29 3
90	+29 3

moval of the solvent under reduced pressure 1.3 gm. of mobile syrup were obtained. On distillation under high vacuum, a forerun of 0.4 gm., which on the basis of the analytical figures was essentially unhydrolyzed trimethyl methylriboside, was obtained. The main fraction (0.8 gm.) distilled at 90–92° at 0.1 mm., having $n_D^{23} = 1.4527$. The substance had the following composition.

4.503 mg. substance: 8 280 mg. CO_2 and 3 380 mg. H_2O
7.696 "   "   : 27.765 " AgI
          $C_8H_{16}O_5$. Calculated. C 50 0, H 8 4, OCH_3 48 5
                 Found.   " 50 2, " 8 4, "   47 7

Its specific rotation was

$$[\alpha]_D^{25} = \frac{+1.69° \times 100}{2 \times 2.152} = +39.3° \text{ (in absolute methyl alcohol)}$$

[9] Willstätter, R., and Schudel, G., *Ber. chem. Ges.*, **51**, 780 (1918).

$$[\alpha]_D^{25} = \frac{+1.72° \times 100}{2 \times 2.12} = +40.6° \text{ (in water)}$$

$$[\alpha]_D^{25} = \frac{+1.84° \times 100}{2 \times 2.12} = +43.4° \text{ (in water with addition of ammonia)}$$

Oxidation of Trimethyl Ribofuranose with Bromine—1.4 gm. of trimethyl ribose were dissolved in 20 cc. of water and 2 cc. of bromine were added in portions of 0.5 cc. during 4 days at 38°. The excess bromine was then removed by aeration, silver oxide was added until all the mineral acid had been neutralized, the mixture was filtered, and the silver salts well washed with hot water. To the combined aqueous solution was added dilute hydrochloric acid from a burette until all the silver had been precipitated. The solution was filtered and the filtrate evaporated to a thick syrup under reduced pressure. The product was heated at 100° for 5 hours at 15 mm. to complete lactonization and then distilled at high vacuum. The main fraction (1.0 gm.) boiled at 90–93° at 0.05 mm. It was a colorless, fairly mobile syrup and had $n_D^{22} = 1.4508$. It had the following composition.

4.325 mg. substance: 7.990 mg. CO_2 and 2.880 mg. H_2O
4.500 "   "   : 16.695 " AgI
$C_8H_{14}O_5$. Calculated. C 50.5, H 7.4, OCH_3 49.0
               Found.   " 50.4, " 7.5, "   49.0

The substance displayed the following specific rotation.

$$[\alpha]_D^{25} = \frac{+1.47° \times 100}{2 \times 1.292} = +56.8° \text{ (in chloroform)}$$

The course of the hydrolysis of the lactone in aqueous solution was studied polarimetrically. The initial specific rotation was

$$[\alpha]_D^{25} = \frac{-0.40° \times 100}{2 \times 1.051} = -19.0°$$

the rotation still being negative after 26 hours.

This has previously been found to be the case with trimethyl-γ-ribonolactone,[4] whereas with trimethyl-δ-ribonolactone[10] the rotation becomes positive before 20 hours have elapsed.

[10] Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **93**, 623 (1931).

p-Toluenesulfonyl Derivative of Monoacetone Ribose—Attempts to prepare a mono-*p*-toluenesulfonyl derivative of monoacetone ribose by using 1 equivalent of *p*-toluenesulfonyl chloride resulted in a very small yield of the crystalline di-*p*-toluenesulfonyl derivative.

1.6 gm. of *p*-toluenesulfonyl chloride were added to a solution of monoacetone ribose (1 gm.) in freshly distilled pyridine (1.5 cc.). The mixture was allowed to stand overnight at 37° and then dissolved in chloroform with the addition of water. The aqueous portion was extracted twice with small quantities of chloroform. The combined chloroform extracts were then washed successively with dilute hydrochloric acid, dilute caustic soda, and water. The chloroform solution was now dried over anhydrous sodium sulfate, filtered, and the filtrate evaporated to dryness under diminished pressure. The product was a pale yellow, very viscous syrup which crystallized from alcohol in white needles, m.p. 122–123°. The substance had the following composition.

4.230 mg. substance: 8.170 mg. CO_2 and 2 040 mg. H_2O
5.245   "   "   : 4.520 " $BaSO_4$
    $C_{22}H_{26}O_9S_2$. Calculated. C 53.0, H 5 3, S 12 9
                Found.   " 52 7, " 5 4, " 11 8

Preparation of Mono-p-Toluenesulfonylmonoiodomonoacetoneribofuranose—A solution of 0.2 gm. of di-*p*-toluenesulfonylmonoacetoneribose in acetone was heated with sodium iodide at 100° for 2 hours. The solution was then evaporated to dryness under diminished pressure, and the dry residue extracted repeatedly with boiling chloroform. The chloroform extracts were washed with dilute sodium thiosulfate solution and with water. After drying the combined extracts over anhydrous sodium sulfate and removing the chloroform, the product (0.2 gm.) was obtained as fine, white needles, melting at 120° on recrystallization from absolute alcohol. The substance had the following composition.

4.671 mg. substance: 6.875 mg. CO_2 and 1 840 mg. H_2O
9.960   "   "   : 5.300 " $BaSO_4$
5.600   "   "   : 2.915 " AgI
    $C_{15}H_{19}O_6SI$. Calculated. C 39 6, H 4.2, S 7.1, I 27.9
                Found.   " 40.1, " 4 4, " 7.3, " 28.1

It had the following specific rotation.

$$[\alpha]_D^{25} = \frac{+0.68° \times 100}{2 \times 0.402} = +84.6° \text{ (in chloroform)}$$

Preparation of Mono-p-Toluenesulfonylmononitromonoacetoneribofuranose—0.2 gm. of the mono-p-toluenesulfonylmonoiodomonoacetoneribofuranose was dissolved in acetonitrile solution containing 0.3 gm. of silver nitrate and boiled under a reflux for 2 hours. The solution was then evaporated to dryness and the dry residue repeatedly extracted with boiling benzene. The benzene was removed under diminished pressure and the product obtained crystalline as long, fine needles. After recrystallization from absolute alcohol it melted at 156°. It had the following composition.

4.490 mg. substance: 7.590 mg. CO_2 and 1.976 mg. H_2O
9.181 "   "   : 0 302 cc. N (30° at 754 mm.)
$C_{15}H_{19}O_9NS$.   Calculated.   C 46.3, H 4.9, N 3 6
              Found   " 46 1, " 4 9, " 3 6

Examination of Crystalline Material (Fraction (1)) from Condensation of Ribose with Acetone—The crystalline material, obtained as the first fraction in the distillation of the condensation product of ribose with acetone, crystallized in hexagonal plates from methyl alcohol and in very long, fine needles from pentane. Both types of crystal melted at 61–62° and had the following composition.

5.220 mg. substance: 10 680 mg. CO_2 and 3 400 mg. H_2O
4.455 "   "   : 9 010 "   "   " 2.830 "   "
12.12 "   "   required (a) 4 70 cc. 0 1 N sodium thiosulfate
12 12 "   "   "   (b) 4.65 "   0 1 "   "   "
$C_8H_{12}O_4$.   Calculated.   C 55 8, H 7 0, (Me_2CO)   33 73
              Found.   " 55 8, " 7 3,   "   (a) 33 75
                        " 55 2, " 7 1,   "   (b) 33 34

It had the following specific rotation.

$$[\alpha]_D^{25} = \frac{-2.08° \times 100}{2 \times 1.616} = -64.35° \text{ (in methyl alcohol)}$$

The results of molecular weight determinations with the Menzies-Wright apparatus are given in Table III.

$C_8H_{12}O_4$.　Calculated mol. wt., 172; found, 171

Hydrolysis of Crystalline Material—0.1167 gm. of the crystalline material was dissolved in 10 cc. of 0.25 N hydrochloric acid and the solution was allowed to stand at room temperature, the course of

TABLE III

*Determination of Molecular Weight by Method of Menzies and Wright**

Weight of substance	Volume of acetone (b.p 56 3°)	Elevation of differential thermometer	Molecular weight
gm	*cc*	*mm*	
0 1921	30	20 5	172 4
0 3811	30	27 5	175 0
0 5448	30	34 5	166 9
Average			171 1

* Menzies, A. W. C., and Wright, S. L., *J. Am. Chem. Soc.*, **43**, 2314 (1921).

TABLE IV

Hydrolysis of Crystalline Material

Time	$[\alpha]_D^{25}$
min	*degrees*
2	−60.4
25	−59 4
60	−57 4
1125	−17.4
1535	−15.8
2675	−15 8

the hydrolysis being followed polarimetrically.　The results are recorded in Table IV.

Condensation of Ribose with Acetone in Presence of Hydrochloric Acid—4 gm. of dry, finely powdered ribose were suspended in 80 cc. of acetone (from the bisulfite compound) containing 0.4 gm. of dry hydrogen chloride and the mixture was shaken for 21 hours at room temperature, after which the solution no longer reduced boiling Fehling's solution.　Isolation was carried out as described

above, except that neutralization was effected with sodium methylate in place of calcium hydroxide.   Yield 3.6 gm.

The condensation product was distilled under high vacuum giving the following fractions: (1) 0.236 gm. of mobile yellow syrup, b.p. 50–60° at 0.07 mm.   (2) 2.566 gm. of fairly viscous syrup, b.p. 105–115° at 0.07 mm.

Fraction 2 was essentially a monoacetone ribose since it had the following composition.

4.435 mg. substance: 8 345 mg. CO_2 and 2 950 mg. H_2O
$C_8H_{14}O_5$.   Calculated.   C 50 5, H 7 4
                Found.   " 51 3, " 7 4

After standing for some days in the refrigerator it partly crystallized.   The crystals were separated and found to melt at 93–94°. This crystalline material had the following analysis.

5 345 mg. substance: 10 915 mg. CO_2 and 3 355 mg. H_2O
$C_8H_{12}O_4$.   Calculated.   C 55 8, H 7 0
                Found.   " 55.7, " 7.0

VARIATIONS IN THE INORGANIC CONSTITUENTS OF MIXED AND PAROTID GLAND SALIVA ACTIVATED BY REFLEX STIMULATION IN THE DOG

By HAMILTON BAXTER

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It was previously found (Baxter, 1929–30, 1931) that marked variations occur in the organic matter, ash, and chlorine of the submaxillary and parotid saliva in normal dogs fed with different substances, as well as in the course of the secretion activated by these substances.   Subsequently some of the inorganic elements of saliva both from the parotid and from the submaxillary glands of normal dogs were investigated to ascertain whether any connection might exist between the type of stimulus used and the level of any given ion in the saliva.   The results of this investigation are presented in this paper.

Most of the previous investigators in this field have confined themselves to acute experiments in which the salivary glands were activated by electrical stimulation of the corresponding secretory parasympathetic nerve or by intravenous injection of massive doses of pilocarpine.   Werther (1886), who used both methods on dogs and rabbits, was one of the first to examine the inorganic compounds of the submaxillary and parotid saliva.   He found in the case of the submaxillary and parotid glands that an increase in the rate of secretion caused a higher concentration of salts in the saliva.   The saliva of each of the three salivary glands investigated contained various amounts of salts.   Werther's figures, *e.g.* for calcium, show that that element was almost twice as abundant in the parotid and sublingual saliva as it usually is in the blood serum. On the other hand, the amount of calcium in the submaxillary saliva was only slightly higher than the average blood serum calcium of the dog.

Gregersen and Ingalls (1931) have shown recently that sodium, but not potassium, in the submaxillary saliva of the dog is depend-

ent upon the rate of secretion resulting from chorda and pilocarpine stimulation. De Beer and Wilson (1932), using pilocarpine as a stimulus in acute experiments on dogs, found that the total amount of carbon dioxide, calcium, and potassium is greater in the parotid saliva than in the blood serum; whereas, there was a smaller concentration of chloride and sodium in the saliva than in the serum. Intravenous injection of solutions of calcium chloride and of sodium carbonate increased the concentration of the corresponding ions in the saliva. The composition of the saliva was not materially affected when concentrated solutions of sodium chloride, potassium chloride, and potassium carbonate were injected into the blood. To this it may be added that Clark and Shell (1927) and Clark and Levine (1927) investigated the inorganic constituents of human "resting" mixed saliva and of mixed saliva obtained under various dietary conditions. The ion concentration of the saliva, they found, differed greatly from that of the plasma, and they could not show any relationship between the concentration of the inorganic constituents of the blood and the amount appearing in the saliva.

Most of the data reported above were obtained through stimulation of the glandular activity by artificial methods. It was recently demonstrated by myself (Baxter, 1932) that pilocarpine is not a true substitute for parasympathetic stimulation of the salivary glands. However, even electrical stimulation of a secretory nerve is not equivalent to the complicated process of reflex stimulation of the gland under normal conditions. It should also be remembered that each gland possesses not one but two secretory nerves. It was therefore decided to study the concentration of different inorganic compounds in the saliva of the parotid and mixed glands, when activated by various stimuli in dogs with permanent salivary fistulæ. In one set of experiments the volume of the saliva secreted in a given time in response to different stimuli was equalized as far as possible. This permitted the rate of secretion of the inorganic parts of the saliva to be estimated independently of the rate of secretion of water.

Methods

Fistulæ of the parotid and mixed glands were made in six normal dogs, some having double parotid fistulæ and others a parotid fis-

tula and fistula of the mixed glands on the same side. Saliva was collected by Pavlov's method. The various stimuli were given to the dog continuously for 5 minutes. The dog was always stimulated three times during each experiment with intervals of from 10 to 15 minutes between each stimulation. Each period of stimulation gave almost identical amounts of secretion. The three samples were combined in order to equalize any slight differences due to factors which could not be controlled. Bread and meat powder were fed continuously from a dish during each 5 minute period. Liquid stimuli were introduced into the mouth cavity by an ingenious device used by Pavlov. A small U-shaped metal tube was attached to the skin of the dog's jaw so that one arm of the tube lay between the buccal fold of the cheek and the teeth as far back as the last molar tooth. The other end of the tube was attached by Mendeleeff's cement to the animal's cheek and connected by a rubber tube with a syringe of 20 cc. capacity, filled with a solution which had to be introduced into the mouth. By movements of the tongue and lips the stimulus was distributed throughout the mouth cavity. The psychic effect of the appliance was very slight. Pilocarpine hydrochloride was injected subcutaneously and the three samples of saliva were collected at the height of the secretory flow. De Beer and Wilson centrifuged their samples of saliva before proceeding with the analysis; in my experiments this was not done. Total solids, organic matter, and ash were determined, as well as chlorine, calcium, total phosphorus, potassium, and the acid-combining power. The total solids were determined by drying at 105° for several hours. The residue was ashed in an electric furnace in which the temperature was controlled by a rheostat. Most constant results were obtained when the platinum crucibles were placed in the furnace when it was cold, the temperature being gradually raised over a period of several hours until all carbon had been eliminated. The ash was dissolved by adding 5 cc. of 0.1 N hydrochloric acid, and was made up to 20 cc. in volumetric flasks. Aliquots were then used for the determination of calcium and potassium. Chlorine was determined by the Wilson and Ball (1928) modification of Van Slyke's method. Calcium was determined in the ash by the Clark and Collip (1925) method, which has been slightly modified for saliva by Clark and Shell (1927). The method of Fiske and Subbarow (1925) was used for

the determination of phosphorus, and that of Kramer and Tisdall (1921) for potassium.   The acid-combining power was determined by the method employed by Pickerell.(1914).

It is evident from an examination of Tables I and II that the saliva, from the same kind of glands, may differ not only in the quantity secreted but also in the concentration of the different ions under the same stimulus in different dogs.   Even when approximately the same volume of secretion was obtained from two dogs under the same stimulus, the constituents of the saliva varied. Furthermore, in the case of some dogs with double parotid fistulæ, although the stimulus was applied equally on both sides of the mouth cavity, the secretion from one side was greater than that from the other.   This peculiarity might have been due to the different way in which each dog distributed the stimulus (solutions or bread and meat powder) about the mouth cavity by movements of the tongue and lips.   When a liquid stimulus was injected on one side only, the secretion from that side was invariably greater than that from the opposite gland, a fact already known. Birukov's (1929) investigation of asymmetrical unconditioned salivary reflexes suggests another explanation of the different responses from paired salivary glands.   According to him, although the endings of the sensory nerves are probably equally distributed on both sides of the mucous membrane of the mouth cavity, the number of such endings connected with the homo- and heterolateral salivary centers may differ in different animals.   If such is indeed the case, this may explain the greater or smaller reaction of paired salivary glands to a stimulus equally distributed over the mouth cavity.

The concentration of ions in the saliva of the parotid gland (Table I) differs from that of the mixed glands (Table II).   The concentration of chlorine and calcium and the acid-combining power are considerably lower in the saliva of the mixed glands as compared with the parotid saliva.   No great difference could be noted in the potassium concentration of either saliva.   The level of total phosphorus was almost always higher in the saliva of the mixed glands.   The source of this phosphorus was not determined. At any rate it was much lower in amount than the inorganic phos-

phorus in dog serum (5 to 6 mg. per cent), showing that little inorganic phosphorus passes from the blood into the saliva. The acid-combining power was markedly lower in the saliva of the mixed glands (17.6 to 31.2 m.-eq.) than in that of the parotid gland (25.2 to 64.4 m.-eq.). Therefore the permeability of the secretory cells of the various salivary glands varies for the different ions.

TABLE I

Response of Parotid Gland to Various Stimuli in Different Dogs

Experiment No.	Date	Dog No.	Stimulus	Volume cc.	Solids per cent	Organic matter per cent	Ash per cent	Cl mg per cent	Ca mg per cent	P mg per cent	K mg per cent	Acid-combining power m.-eq
1	May 10	5	Bread and meat powder	66 4	1 59	0 78	0 81	312	22 8	1 7	53 1	49 2
2	Feb. 15	4	" "	34 5	1 52	0 63	0 89	309	24 4	2 5	63 2	66 2
3	June 22	8	" "	33 9	1 24	0 51	0 73	265	25 9	1 8	78 1	56 6
4	Apr. 6	4	NaCl 5%	38 5	1 22	0 39	0 83	284	26 0	1 4	68 0	64 0
5	" 15	5	" 5%	33 3	1 03	0 30	0 73	301	20 0	0 9	45 7	38 8
6	June 27	7	" 5%	30 3	1 12	0 36	0 76	304	25 1	0 9	39 6	41 6
7	Apr. 22	5	HCl 0 25%	70 0	1 45	0 65	0 80	282	23 6	2 0	56 7	52 0
8	Feb. 19	4	" 0 25%	38 3	1 34	0 49	0 85	294	25 8	1 9	62 2	64 4
9	Mar. 4	6	" 0 25%	24 4	1 47	0 68	0 79	271	24 8	1 9	68 7	62 8
10	Apr. 23	5	Meat extract 10 per cent	40 3	1 10	0 50	0 60	295	20 4	0 9	37 9	25 2
11	Feb. 17	4	" "	33 8	1 14	0 39	0 75	328	26 6	0 7	63 9	49 6
12	" 25	6	" "	16 0	1 55	0 80	0 75	301	25 2	1 3	73 8	48 8
13	Mar 2	6	Milk	3 8	1 47	0 93	0 54	170	23 9		91 6	42 4
14	Feb. 20	4	"	5 1	1 30	0 65	0 65	309	20 9	1 3	76 0	37 6
15	Mar. 5	4	Pilocarpine 20 mg.	33 4	1 13	0 31	0 82	260	21 2	0 8	25 0	60 8

The effect of different stimuli upon the parotid glands of five dogs may be seen in Table I. Some slight variations in the ionic concentration in different samples of saliva, notwithstanding equal secretion of water, could be noted in the experiments with 5 per cent NaCl and 0.25 per cent HCl solutions and the meal of bread and meat powder (*cf.* Experiments 2, 4, and 8 on Dog 4). It would

not be quite correct, however, to refer all these variations, small as they are, to the inevitable errors of the methods.

Interesting results were obtained on a dog with a double parotid fistula (Table III). It may be seen that the difference in the concentration of certain ions was greater in saliva obtained by different stimuli from the parotid gland on one side than in saliva secreted by both parotid glands in response to the same stimulus.

TABLE II

Response of Mixed Glands to Various Stimuli in Different Dogs

Experiment No.	Date	Dog No	Stimulus	Volume	Solids	Organic matter	Ash	Cl	Ca	P	K	Acid-combining power
				cc	per cent	per cent	per cent	mg per cent	mq per cent	mg per cent	mg per cent	m-eq.
1	May 12	2	Bread and meat powder	45 7	1 61	1 14	0 47	134	12 7	1 5	66 9	31 2
2	" 13	2	" "	42 3	1 53	1 08	0 45	136	12.2	1 6	70 1	30 4
3	" 10	5	" "	61 4	1 38	0 78	0 60	238	9 8	1.9	64 3	26 8
4	" 11	5	" "	62 4	1 41	0 82	0 58	240	10 2	1 8	66 0	26 0
5	Apr. 29	2	NaCl 5%	24 8	0 53	0 19	0 34	98	7.4	3 0	48.1	29 2
6	May 16	2	" 3%	10 6	0 44	0 15	0.29	63	7 0	2 6	66 3	21 6
7	Apr. 15	5	" 5%	55 8	0 75	0 24	0 51	219	6 5	1 2	49 1	22 0
8	May 3	2	HCl 0 25%	35 7	0 81	0.41	0 40	142	8.1	2 5	54 8	32 0
9	Apr. 22	5	" 0.25%	63 0	1 19	0.58	0.61	246	8 8	2 1	59 6	23 2
10	May 18	2	Meat extract 10 per cent	14 2	0 50	0 21	0 29	58	5 8	1 5	83 5	20 0
11	Apr. 23	5	" "	48 0	0 82	0.35	0.47	207	7 6	2 2	49 7	17 6
12	May 17	2	Milk	7 0	1.38	1.00	0.38	58	13 2		92.5	24 0

Thus one gland served as a control for the other. These small variations of ionic concentration in different samples of saliva might also be the result of some general condition of the animal on the day of each particular experiment (*e.g.* state of nutrition, panting, etc.). As de Beer and Wilson (1932) showed, the increased concentration in the blood of calcium chloride and sodium carbonate is reflected in the composition of saliva in relation to these two ions. Besides these slight variations in the concentration of ions in saliva activated by the introduction into the mouth of the solutions of

TABLE III

Dog 6 with Two Parotid Fistulæ

Date	Stimulus	Right parotid									Left parotid								
		Volume	Solids	Organic matter	Ash	Cl	Ca	P	K	Acid-combining power	Volume	Solids	Organic matter	Ash	Cl	Ca	P	K	Acid-combining power
		cc	per cent	per cent	per cent	mg per cent	mg per cent	mg per cent	mg per cent	m-eq	cc	per cent	per cent	per cent	mg per cent	mg per cent	mg. per cent	mg per cent	m-eq
Mar.																			
17	NaCl 5%	26 7	1 19	0 39	0 80	257	26 1	1 2	59 1	64 0	24 1	1 21	0 40	0 81	260	26 1	1 2	56 1	63 2
7	HCl 0.125%	26 5	1 33	0 53	0 80	284	25 6	1 5	61 3	62 0	22 7	1 36	0 56	0 80	282	25 2	1 5	60 0	61 2
14	Bread and meat powder	27 7	1 60	0 75	0 85	271	24 0	1 7	60 9	64 0	24 5	1 65	0 80	0 85	274	24.5	1 7	63 3	65 2

sodium chloride, hydrochloric acid, or a meal of bread and meat powder, there were very striking differences in the secretion from the same Dog 4 (Table I) in response to meat extract (Experiment 11) and to pilocarpine (Experiment 15), the samples being taken at the height of the secretion.   The volume of the secretion for both stimuli during the same period of time (15 minutes) was practically the same, *viz.* 33.8 cc. and 33.4 cc.   There was an insignificant difference in the concentration of phosphorus in the two samples (0.7 and 0.8 mg. per cent).   The acid-combining power was lower in the meat extract saliva (49.6 m.-eq.) than in the pilocarpine saliva (60.8 m.-eq.).   All other ions, especially the potassium ion, were much lower in pilocarpine saliva than in meat extract saliva (Cl, 328 and 260 mg. per cent; Ca, 26.6 and 21.2 mg. per cent; K, 63.9 and 25.0 mg. per cent).   Thus the two stimuli activated an equal flow of water through the glandular cells during the same period of time, but quite a different amount of inorganic substances was secreted in each case.   Analogous relations may be noted if the pilocarpine secretion (Experiment 15) is compared with the secretion on bread and meat powder (Experiment 2, Dog 4).

It is interesting to compare with these data the composition of the saliva in the same Dog 4 (Table I) on milk (Experiment 14). The volume of the secretion on milk (5.1 cc.) was only about one-sixth the amount of that obtained on meat extract and pilocarpine. Nevertheless, it was not merely a diminution or increase in the concentration of ions that was to be noted in this saliva, but a special ion content, typical for the given stimulus.

The same slight variations in the ion concentration, which could not be explained by the errors of the method alone, were noted in the mixed gland saliva secreted under different stimuli (Table II, *cf. e.g.* Experiment 4 (meal of bread and meat powder) with Experiment 9 (0.25 per cent HCl) on Dog 5).   The volume of the secretion during 15 minutes (62.4 cc. and 63.0 cc. respectively) was practically the same in both experiments.   Similar results may be seen also in two experiments (Experiments 6 and 10) on Dog 2 (Table II) in which saliva was obtained by stimulation with 3 per cent NaCl, and by the introduction of 10 per cent meat extract into the mouth.   On the other hand, the figures for the ion concentration in the samples of saliva secreted in response to the *same* stimulus in approximately the same volume of water are remarkably

close to each other (*cf.* Dog. 2, Experiments 1 and 2; Dog 5, Experiments 3 and 4). Again, the mixed glands in response to a meal of milk produced a secretion of saliva with quite a different ionic concentration—*cf.* Experiment 6 (3 per cent NaCl) and Experiment 12 (milk). The concentration of calcium and potassium was far higher in the case of milk than in the case of NaCl. This difference cannot be explained by the different rate of secretion of the saliva in the two experiments because it was insignificant. These experiments show that there are certain variations in the inorganic composition of the saliva from the parotid and mixed glands, when activated by different stimuli. These variations, which are rather small following certain stimuli and more marked under other stimuli, occur independently of the rate of secretion of the saliva.

The next problem which we investigated was the influence of the strength of the stimulus on the concentration of different ions in the saliva of the mixed and parotid glands. Only a few investigators have studied the influence of the rate of secretion on the concentration of the different ions in saliva. According to Werther (1886) the concentration of water-soluble salts (NaCl, NaHCO₃) rises with the increased rate of secretion of the submaxillary saliva activated by chorda tympani stimulation in the dog. The water-insoluble salts do not always follow this rule. Different relations were observed in the parotid saliva of a rabbit in which the flow was stimulated by pilocarpine. With the increase in the rate of secretion the concentration of the water-insoluble salts greatly diminished, whereas the concentration of water-soluble salts was practically unchanged. Gregersen and Ingalls (1931) showed that the sodium concentration in dog submaxillary saliva obtained under chorda or pilocarpine stimulation rose with the increase in the secretion rate, but the potassium concentration under these circumstances remained practically constant. From control experiments of de Beer and Wilson (1932) it may be seen that, when the rate of the pilocarpine salivary secretion from the dog's right parotid gland was doubled (0.26 cc. and 0.5 cc. per minute in two consecutive samples, Samples 2 and 3, Table I), the Cl, K, and Ca concentration fell a little, whereas the Na concentration rose somewhat.

There was no mention in the literature of any determinations

of the concentration of different ions in the saliva of normal dogs obtained by stimulation of the mouth cavity with various substances in solutions of different strength.　In Table IV are shown experiments in which diminishing concentrations of NaCl and HCl are used to stimulate the mouth cavity in a dog with permanent fistulæ of the mixed and parotid glands.　The amount of fluid injected into the dog's mouth was the same in each experiment, but the concentration varied.　The experiments with the solutions of NaCl and HCl in various concentrations were performed on different days, the secretions of saliva from the mixed and parotid glands in each experiment being collected simultaneously and analyzed. Of the two stimuli employed hydrochloric acid stimulated the parotid gland in this animal to a more profuse production of saliva than the submaxillary gland.　It is interesting to note that the total ash is always lower in the saliva of the mixed glands, even when the secretory rate is the same or greater.　In these experiments, as in those reported above (Table II) the values for chlorine, calcium, and acid-combining power were invariably lower in the submaxillary saliva.　On the other hand, the concentrations of potassium and phosphorus were usually somewhat higher in the saliva of the mixed glands than in the parotid saliva, independently of the rate of secretion.　As the strength of the stimulus was diminished, the secretion of water was also curtailed until, as in the case of 1 per cent NaCl, the salivary flow reached an extremely low level.　The concentration of organic matter and total ash also fell with the diminution of the stimulus, except in the case of 1 per cent NaCl, where only a few cc. of saliva were secreted during each 5 minute period.　If the secretion is too scanty there may be some increase in the content of organic substances and other constituents of the saliva, because very weak impulses from the center fail to activate an adequate flow of water.

There are definite changes in the concentration of different ions in saliva obtained by stimulation of the animal with NaCl and HCl solutions of various strength.　In the saliva of the mixed glands the concentration of Cl and increase of the acid-combining power depended on the rate of secretion.　The potassium concentration underwent certain insignificant variations which were independent of the rate of secretion.　The calcium ion remained practically constant under all conditions.

TABLE IV

Response of Mixed and Parotid Glands to Stimuli of Decreasing Strength (Dog 5)

Saliva was collected during three periods of 5 minutes each, with intervals between each stimulus varying from 10 to 15 minutes.

Experiment No.	Date	Stimulus during each 5 minute period	Mixed glands									Parotid gland								
			Total volume in 15 min, cc	Solids, per cent	Organic matter, per cent	Ash, per cent	Cl, mg per cent	Ca, mg per cent	P, mg per cent	K, mg per cent	Acid-combining power, m.-eq.	Total volume in 15 min, cc	Solids, per cent	Organic matter, per cent	Ash, per cent	Cl, mg per cent	Ca, mg per cent	P, mg per cent	K, mg per cent	Acid-combining power, m.-eq.
1	May 6	60 cc. 10% NaCl	55.7	0.910	0.380	0.53	253	7.7	1.2	2.55	2.20	468	1.240	0.470	0.77	298.23	0	1.3	3.53	452.0
2	Apr. 15	60 " 5% "	55.8	0.750	0.240	0.51	219	6.5	1.2	2.49	1.22	0.33	3.1	0.300	0.73	301.20	0	0	9.45	738.8
3	May 4	60 " 3% "	36.6	0.500	0.200	0.30	125	7.7	1.1	1.54	1.12	4.24	0.620	0.190	0.43	203.19	4	0	4.42	719.2
4	" 5	60 " 1% "	4.2	0.620	0.270	0.35	136	7.4			14.0	0.1	0.940	0.300	0.64	275				20.4
5	Apr. 22	60 " 0.25% HCl	63.0	1.190	0.580	0.61	246	8.8	2.1	59.6	23.2	2.70	0.450	0.650	0.80	282.23	6	2	0.56	752.0
6	" 25	60 " 0.125% "	41.5	0.880	0.390	0.49	217	8.5	1.9	49.7	18.8	8.69	0.390	0.580	0.81	343.23	4	1	51.9	944.0
7	" 28	60 " 0.062% "	26.5	0.560	0.280	0.28	102	8.0	1.7	55.7	14.0	0.58	51.0	51.0	0.75	295.22	0	1	3.53	044.0

In the parotid saliva Cl concentration did not show such marked and regular dependence on the rate of secretion as in the saliva of the mixed glands. The acid-combining power diminished with the diminution of the secretion. The potassium concentration practically depended on it but the calcium concentration remained constant. If the volume of the secretion and the concentration of ions in the saliva activated by NaCl and HCl respectively are compared, it may be seen that for the mixed glands the different solutions of NaCl are equal or even somewhat stronger stimuli than different solutions of HCl. On the other hand, for the parotid gland only the strongest concentrations of NaCl (10 per cent) and HCl (0.25 per cent) used gave comparable secretions. The volume of the secretion, and the concentration of organic material and of different ions rapidly decreased with the diminished concentration of NaCl, and underwent only very small changes when weaker concentrations of HCl were employed. Thus each gland responds with a specific reaction to different stimuli.

DISCUSSION

The principal fact established in this investigation is that the rate of the secretion is not the sole factor which determines the inorganic composition of parotid and mixed gland saliva. To a certain degree the ionic concentration of saliva is influenced by the nature of the stimulus acting on the receptive surface of the mouth cavity. Since the greater part of the inorganic constituents of saliva is derived by the secretory cells from the blood, the experiments reported above indicate that the permeability of the gland, though practically always the same for water, varies for the different ions according to the type of stimulus applied to the receptive surface. This fact may be interpreted in two different ways.

It may be supposed that when various stimuli act on the mucous membrane of the mouth cavity, they are transmitted by a special nervous process to the salivary center and may activate in the efferent neurones impulses of qualitatively different types. The secretory cells will thus receive certain nervous impulses which will make them permeable for water and for a definite combination of ions in each case, depending on the stimulus used. Although this theory cannot be entirely excluded (Babkin, 1913; Hitzker, 1914; Mansfeld, Hecht, and Kovács, 1929–30, 1931),

another interpretation seems more probable (Babkin, 1931). It may be supposed that each stimulus acts reflexly on a definite combination of the various parts of the salivary center which regulate the work of different sets of the secretory epithelia composing the salivary glands. Certain facts support the latter theory. Thus the inorganic composition of dog saliva differs markedly in the case of the different salivary glands, showing that the various kinds of epithelia from which these glands are formed possess different degrees of permeability for the ions in the blood. There may be slight variations in the inorganic composition of the saliva in different dogs in response to one and the same stimulus. These variations might possibly be due to some individual differences in the structure of the glands or in the metabolism or in the composition of the blood in different dogs. Although in their histological structure the parotid gland and pancreas have many features in common, the inorganic contents of their secretions are quite different (de Beer and Wilson, 1932; Ball, 1930; Johnston and Ball, 1930). The changes in the inorganic composition of the saliva in response to stimuli of different strengths depend not only on the rate of the secretion but also on the specific properties of the stimulus (*cf.* Table IV). This indicates that different processes are activated in each case.

Histological observations show that, in the interlobular ducts (*Speichelröhren*) of the salivary glands, are epithelia which secrete chiefly water (Mislawsky and Smirnow, 1896). According to Boggino (1931) iron, mercury, and bismuth salts are excreted chiefly by the epithelium of the "secretory-excretory" ducts of these glands. Therefore, it may be supposed that various stimuli, acting on the mucous membrane of the mouth cavity, activate reflexly certain, particular glandular elements of the salivary glands, or all of such elements but in various degrees. Each of these sets of epithelia has its own specific degree of permeability for the inorganic constituents of the blood.

One other point should be mentioned in connection with the reflex secretion of saliva and its inorganic composition. In these experiments (see Table I), as well as in the previous investigation already referred to above (Baxter, 1932), the particular nature of the pilocarpine secretion was revealed. The chlorine, calcium, and potassium concentrations were much lower in the pilocarpine

parotid saliva than in the saliva secreted at the same rate on meat extract or a meal of bread and meat powder.   A particularly low figure was obtained for potassium (25.0 mg. per cent in "pilocarpine" saliva against 63.9 mg. per cent in "meat extract" saliva). The acid-combining power was somewhat higher in the former than in the latter.   This shows that pilocarpine must be considered as a special, artificial stimulus which provokes a particular activity of the salivary glands differing from that activated by normal reflex action.

SUMMARY

1. A study was made of the inorganic composition of saliva obtained from the parotid and mixed glands by reflex stimulation and subcutaneous injection of pilocarpine in dogs with permanent salivary fistula.

2. The concentration of chlorine and calcium and the acid-combining power are considerably lower in the saliva of the mixed glands than in the parotid saliva.   No great difference could be observed in the potassium concentration of either saliva, but the concentration of phosphorus was somewhat higher in the saliva of the mixed glands.

3. Different stimuli acting on the receptive surface of the mouth cavity may produce from either the parotid or the mixed glands a flow of saliva of equal volume but of different inorganic composition.   Under some stimuli these variations are very small, under others quite marked.

4. By diminishing the strength of solutions of NaCl and HCl introduced into the mouth, the volume of the secretion is also diminished.   The reaction of the mixed glands to NaCl is stronger than to HCl; the reverse is the case in the parotid gland.

5. When the strength of the stimulus was diminished, the concentration of chlorine and the acid-combining power fell.   No changes or only very slight ones were noted in the concentration of calcium and potassium ions under these conditions.

6. The chlorine, calcium, and potassium concentrations were much lower in the parotid saliva activated by pilocarpine than in the parotid saliva secreted at the same rate on meat extract or bread and meat powder, while the acid-combining power was equal in the two salivas, or somewhat higher in the saliva activated by pilocarpine.

I wish to thank Professor B. P. Babkin for his helpful criticism and advice in directing this investigation.

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THE CHEMISTRY OF THE LIPIDS OF YEAST

I. THE COMPOSITION OF THE ACETONE-SOLUBLE FAT*

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Although a large number of publications are recorded on the chemical composition of yeast, very few reports are found dealing with the lipids. Early work reported by Naegeli and Loew (1), Gerard and Darexy (2), Hinsberg and Roos (3), Neville (4), and MacLean and Thomas (5) would indicate that yeast contains both saturated and unsaturated fatty acids The presence of palmitic, stearic, and arachidic acids has been established as well as that of oleic, linoleic, and an unsaturated acid in the C_{16} series. Other fatty acids such as butyric, lauric, a saturated C_{24} acid, and various oxygenated acids have been reported but without very definite proof of their identities. Evidence has also been presented by Weichherz and Merlander (6) and by Weiss (7) for the presence of an optically active valeric acid.

In order to secure more information concerning the composition of the alcohol-ether-soluble constituents of living yeast, we have devoted some time to the study of yeast lipids. Moreover, we were interested in discovering whether the fat from a unicellular organism such as yeast contained any acids similar to tuberculostearic or phthioic acids found in tubercle bacilli.

Through the cooperation of The Fleischmann Laboratories, New

York, we were provided with large quantities of fresh living moist yeast. The lipids were extracted with mixtures of alcohol and ether and were separated into phospholipids and acetone-soluble fat. In this paper we report upon the general procedures employed and upon the composition of those lipids soluble in cold acetone.

The results obtained indicate that the lipids of yeast are very similar in composition to the fats found in ordinary plants and animals. The neutral fat is composed of mixed glycerides of saturated and unsaturated fatty acids; the unsaturated acids representing about 80 per cent of the total acids. No saturated liquid fatty acids similar to phthioic acid (8) are present. By judging from the acetyl value, a small amount of hydroxy acids was at hand, but we were unable to isolate any such acid. The fat obtained by our method of extraction from living yeast cells contains only a very small amount of volatile fatty acids and we did not find even a trace of the valeric acid referred to by Weichherz and Merlander (6) and by Weiss (7).

A thorough search was made for an acid of a higher molecular weight than stearic acid, such as the arachidic acid reported by Neville (4) and by MacLean and Thomas (5), but we were unable to find any such acid in any of the lipid fractions.

<div align="center">EXPERIMENTAL</div>

The fresh moist yeast (*Saccharomyces cerevisiæ*) was supplied in 10 or 15 pound lots as needed by The Fleischmann Laboratories. The yeast was grown for a period of 8.5 hours in a thoroughly aerated molasses medium containing ammonium phosphate. It was then collected by centrifugation, cooled, and pressed.

The extraction of the lipids as well as other operations was carried out according to the methods used in this laboratory in investigations of the lipids of acid-fast bacteria (9). The air was always excluded as much as possible by using an atmosphere of carbon dioxide or nitrogen and all solvents were freshly distilled and saturated with an inert gas before use. In every case in which filtrations or decantations were made, the insoluble material was washed with an appropriate solvent. For the sake of brevity these obviously necessary operations are not mentioned.

Extraction of Lipids—The yeast was first partly dehydrated by digestion with alcohol after which it was filtered and treated twice

at room temperature for 2 days with mixtures of alcohol and ether. The extracts were combined and concentrated under reduced pressure. The lipids were extracted from the aqueous residual suspension with ether. After drying, the ethereal extracts were filtered through a Chamberland candle filter and the solvent removed. The dark oil thus obtained weighed 1063 gm. or 6.02 per cent of the dry yeast.

The extracted yeast was further treated with alcohol containing 1 per cent of hydrochloric acid for 2 days at a temperature of 30–35°. By this extraction a further 150 gm. of a dark ether-soluble oil was obtained and designated acid-extraction fat.

Additional extractions with strong acids, chloroform, or other solvents yielded only insignificant amounts of ether-soluble materials. The yeast was therefore dried in the air and stored in bottles. A total of 60 kilos of moist yeast was extracted and the air-dried residue weighed 16.4 kilos.

Separation of Lipid Fractions—The crude lipids were dissolved in 1 liter of absolute ether and 2 volumes of acetone were added. The phospholipids separated as a sticky mass and, after cooling, the supernatant liquid was decanted. This solution, after it had been concentrated until the ether was removed, was again cooled and decanted from an insoluble oily product. The final solution was concentrated to dryness and the residue, which weighed about 700 gm., was designated *acetone-soluble fat.*

The oily substance mentioned above was dissolved in ether and the solution treated with 2 volumes of acetone. A sticky mass which separated was collected and added to the original phospholipid fraction. The ether-acetone solution on evaporation to dryness left an oily residue weighing about 150 gm., which was designated *acetone-insoluble fat.*

Purification of Phospholipids—The phospholipids were dissolved in ether and precipitated by adding 2 volumes of acetone. The supernatant solution was decanted and the precipitated material redissolved in ether and again separated by the addition of acetone. After repeating these operations twenty times, the phospholipids were collected and dried *in vacuo.* The material was a brownish mass weighing 154 gm. The mother liquors were concentrated to dryness and the small residue added to the acetone-insoluble fat.

For analysis the phospholipids were dried to constant weight at 56° *in vacuo* over phosphorus pentoxide. The dried preparation was brittle and very hygroscopic.

0.2096, 0.2231 gm. substance: 0.0314, 0.0340 gm. $Mg_2P_2O_7$
0.5452, 0.6652 "   "   (Kjeldahl): 6.85, 8.55 cc. 0.1 N HCl
0.4047 gm. substance gave 0.0370 gm. ash
                  Found: P 4.17, 4.24; N 1.75, 1.80; ash 9.14

The ratio of nitrogen to phosphorus is as 1 : 1.07.

The composition of the phospholipids will be reported in a separate paper.

Acetone-Soluble Fat. Analytical Constants—The following constants of the fat were determined according to the official methods (10).

Saponification No....	109 6
Free acid No.	28 6
Iodine No. (Hanus).	61 3
Reichert-Meisel No.	2 3
Polenske No. ...	0 5
Acetyl No. .	20 2*
Unsaponifiable matter, *per cent*	46 7

* The acetyl number was determined after the fat had been standing in a closed flask for about 2 years. It is possible therefore that a certain amount of oxygenated acids may have been formed by oxidation of the unsaturated acids during this time.

Saponification—A portion of the fat weighing 201.3 gm. was saponified by refluxing for 1 hour with an excess of alcoholic potassium hydroxide after which the solution was diluted with water and extracted with ether. The ether was distilled off and the residue was again refluxed with alcoholic potassium hydroxide, the unsaponifiable matter being extracted with ether as before. A light yellow oil weighing 92 gm., or 45.6 per cent of the fat, composed this fraction.

The fatty acids were extracted with ether after the soap solution had been acidified with hydrochloric acid. The acids formed a thick oily mass weighing 95.6 gm. or 47.4 per cent of the fat.

For the isolation of the water-soluble constituents, the acidified solution, after extraction of the fatty acids, was evaporated to dryness under reduced pressure and extracted in the usual manner

with absolute alcohol. The yield of crude glycerol was 11.9 gm. or 5.9 per cent of the fat.

Unsaponifiable Matter—The unsaponifiable matter when tested by the Hanus method was found to have an iodine number of 7, thus indicating that the bulk of the material consisted of saturated compounds. A small amount of crystalline sterols which separated from the oil on standing was removed by filtration and was not further examined.

In order to determine the chemical composition of the oily portion of the unsaponifiable matter, it was necessary to remove the sterols completely. This object was successfully accomplished by the application of a modified Liebermann-Burchard reaction. A similar procedure has been used by Windaus and Resau (11) for the separation of saturated from unsaturated hydrocarbons in the cholesterol series and by Anderson and Nabenhauer (12) for the separation of saturated from unsaturated sterols.

The reaction was carried out in a separatory funnel. About 25 gm. portions of the oil were dissolved in 250 cc. of carbon tetrachloride, 50 cc. of acetic anhydride were added, and then 15 cc. of concentrated sulfuric acid were added drop by drop with shaking and cooling. The deep green reaction mixture was carefully diluted with a little water and allowed to stand until a dark colored acid layer separated on top. The nearly colorless carbon tetrachloride layer was drawn off, washed with dilute alkali and water, and dried over sodium sulfate. A light yellow oil was obtained on removing the solvent. The oil gave no sterol color reactions and did not absorb any iodine when tested with Hanus solution.

This oil was separated by fractional distillation at very low pressures into a series of fractions ranging in boiling point from 72–200°. The various fractions were repeatedly refractionated without yielding any clear cut pure fractions. All distillates were colorless oils. The apparently purest cuts were analyzed and found to be saturated hydrocarbons corresponding to the general formulas C_nH_{2n} and C_nH_{2n-2}. The composition and molecular weight of the lowest fraction corresponded to the formula $C_{19}H_{38}$, a middle fraction to $C_{30}H_{60}$, and the highest to $C_{34}H_{66}$.

Since it appeared improbable that living cells would produce such a large amount of their ether-soluble constituents in the form of saturated cyclic and bicyclic hydrocarbons, we examined the

unsaponifiable matter prepared from a small lot of yeast grown under carefully controlled conditions in The Fleischmann Laboratories, New York.   In this case we found the unsaponifiable matter to consist entirely of crystalline sterols.   We conclude, therefore, that the hydrocarbons described above were not derived from the yeast fat but were present as accidental contaminations.   In this connection it is interesting to note that Daubney and MacLean (13) mention a yellow saturated oil as occurring in large amount in the unsaponifiable matter of yeast fat.   It is likely that this oil was introduced accidentally into their yeast in the same unknown manner in which it was introduced into the yeast used in this investigation.

Examination of Fatty Acids—The mixed fatty acids formed a semisolid mass with an iodine number of 102.   They were separated by means of the lead soap-ether treatment (14) into 74.9 gm. or 78 per cent of liquid acids and 17.4 gm. or 18 per cent of solid acids.

Liquid Acids—The iodine number (Hanus) of the liquid acids was 119.   After catalytic reduction (15) the acids were again separated by means of the lead soap-ether procedure (14) into 56.7 gm. of solid reduced acids and 7.4 gm. of liquid acids.   The latter material still had an iodine number of 66.   Several attempts were made to reduce this product after thorough purification but the final acid still had an iodine number of 52.

A larger quantity of mixed acids obtained from yeast secured from The Fleischmann Laboratories was examined in the manner described above.   In this case also a small amount of liquid fatty acids, having an iodine number of 55, was obtained.   These also could not be further reduced by catalytic reduction.   While the nature of the unreducible fraction of the liquid acids could not be determined, it is evident that the acetone-soluble fat of yeast does not contain any liquid saturated fatty acids analogous to those obtained from acid-fast bacteria.

General Procedure for Examination of Fatty Acids—The fatty acids were converted into their methyl esters and the latter were carefully fractionated at pressures varying from 0.002 to 0.005 mm. For these fractions the melting point and index of refraction at 55° were taken as criteria of purity.   The purest fractions were then saponified and the free acids recrystallized.   The melting point

and mixed melting point with the corresponding authentic acid were then taken and the molecular weight determined by titration of an alcoholic solution of the acid with 0.1 N alcoholic potassium hydroxide.   Finally, from a consideration of the amounts and the indices of refraction of all the ester fractions, the percentage composition of the esters, and hence the acids, was approximated.

Solid Fatty Acids – A small amount of a low-boiling ester was obtained.   This melted at 19–20° and on saponification yielded an acid melting at 53.5–54.5°.   A mixed melting point with myristic acid showed a depression of 3.5°.   The molecular weight was 236 as compared with 228 for myristic acid.   Hence this acid is probably a mixture of palmitic with some lower acid, possibly lauric. The amount obtained was too small to permit of complete identification.

Two pure ester fractions were obtained and were shown to be methyl palmitate and methyl stearate by the following data.

Fraction I - M. p. 28-29°; $n_D^{55} = 1.4260$; free acid m. p. 62–63°.

0.8821 gm. substance required 34.26 cc. 0.1 N KOH
$C_{16}H_{32}O_2$.   Calculated mol wt., 256; found, 257

Fraction II —M. p. 38–39°; $n_D^{55} = 1.4300$; free acid m. p. 71–71.5°.

0.8156 gm. substance required 28.49 cc. 0.1 N KOH
$C_{18}H_{36}O_2$.   Calculated mol. wt., 284; found, 286

The indices of refraction at 55° were determined for methyl palmitate and methyl stearate and found to be 1.4260 and 1.4300 respectively.

The solid fatty acids were composed of about 75 per cent palmitic acid and 25 per cent stearic acid.

Solid Reduced Acids—Two pure ester fractions were obtained and were shown to be methyl palmitate and methyl stearate by the following data.

Fraction I—M. p. 28-29°; $n_D^{55} = 1.4260$; free acid m.p. 62–63°.

0.6503 gm. substance required 25.27 cc. 0.1 N KOH
$C_{16}H_{32}O_2$.   Calculated mol. wt., 256; found, 257

Fraction II—M. p. 38–39°; $n_D^{55} = 1.4300$; free acid m. p. 71–71.5°.

0.8847 gm. substance required 31.24 cc. 0.1 N KOH
$C_{18}H_{36}O_2$.   Calculated mol. wt., 284; found, 283

These acids were composed of about 25 per cent palmitic acid and 75 per cent stearic acid.

Examination of Acetone-Insoluble Fat and Acid-Extraction Fat— With regard to the presence of an acid of higher molecular weight than stearic acid, the acetone-insoluble fat and the fat obtained by extracting the cell residues with alcohol containing 1 per cent of hydrochloric acid were also examined.

The acetone-insoluble fat had an iodine number of 42. About 45 gm. of fat were saponified and worked up in the manner described above for the acetone-soluble fat. The unsaponifiable matter amounted to 24.7 gm. or 56 per cent, the fatty acids to 17.4 gm. or 39 per cent, and the water-soluble fraction to 2 gm. or 4.5 per cent. The fatty acids were separated by the lead soap-ether treatment (14) into 1.6 gm. of solid acids and 14.9 gm. of liquid acids having an iodine number of 105.

The acid-extraction fat had an iodine value of 54. About 65 gm. of fat were saponified as above, yielding 23.9 gm. or 37 per cent of unsaponifiable matter, 31.4 gm. or 49 per cent of fatty acids, and 9.2 gm. or 14 per cent of water-soluble constituents. The fatty acids were separated as above into 2.9 gm. of solid acids and 27.3 gm. of liquid acids having an iodine number of 101.

The solid acids from these two sources were combined, converted into methyl esters, and separated by fractional distillation under highly reduced pressure into two fractions. The lower boiling fraction weighed 2.5 gm., melted at 24° and $n_D^{55} = 1.4285$. The higher boiling fraction weighed 1.36 gm., melted at 30° and $n_D^{55} = 1.4307$. On saponification, this fraction yielded an acid melting at 67° after several recrystallizations. The molecular weight was 288. It is evident from the above data that no appreciable amount of an acid higher than stearic acid is present.

Identification of Glycerol —The crude glycerol mentioned before was a dark colored thick syrup. When a portion was heated with acid potassium sulfate, the characteristic odor of acrolein was noticed. The syrup did not give the Molisch test for carbohydrates and did not reduce Fehling's solution either before or after boiling with dilute acid. At a pressure of 2 to 3 mm. the syrup distilled at 144–146° giving a light yellow distillate. 1 gm. of this syrup was benzoylated according to the method of Einhorn and Hollandt (16). The reaction product was recrystallized from

absolute methyl alcohol.   Beautiful prismatic needles were obtained which melted at 75–76° and showed no depression of the melting point when mixed with authentic glycerol tribenzoate. The results indicate that glycerol is the chief water-soluble constituent of the acetone-soluble fat of yeast.

In conclusion we acknowledge with pleasure our indebtedness to Dr. C. N. Frey of The Fleischmann Laboratories, New York, for supplying the yeast used in this investigation.

Anyone interested in further details of this work may consult the thesis by M. S. Newman on file in the Yale University Library.

SUMMARY

Fresh living yeast was extracted with alcohol and ether.   The lipids obtained amounted to 6.02 per cent calculated on the dried yeast as a basis.   Further extraction of the partly defatted cells with alcohol containing 1 per cent of hydrochloric acid yielded material amounting to 0.86 per cent calculated on dry yeast.

The alcohol-ether-soluble lipids were separated into phospholipids, acetone-insoluble fat, and acetone-soluble fat.

The acetone-soluble fat was saponified and the following substances obtained: sterols, a mixture of saturated cyclic and bicyclic hydrocarbons ranging from $C_{19}H_{34}$ to $C_{31}H_{66}$ present as an impurity in the yeast used, glycerol, and fatty acids.   The saturated acids consisted of about 75 per cent of palmitic and 25 per cent of stearic acid, together with a trace of some acid lower than palmitic acid. The unsaturated acids on catalytic reduction gave a mixture of about 25 per cent of palmitic and 75 per cent of stearic acid.

No appreciable amount of an acid higher than stearic acid was found in the acetone-insoluble fat or in the fat obtained by extraction of the yeast cells with alcohol containing 1 per cent of hydrochloric acid.

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THE CHEMISTRY OF THE LIPIDS OF YEAST

II. THE COMPOSITION OF THE PHOSPHOLIPIDS*

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In connection with the investigations conducted in this laboratory during the past several years on the lipids of tubercle bacilli and related acid-fast bacteria (1), we have become interested in the composition of similar fractions isolated from other unicellular organisms. The present report deals with the chemical composition of the phospholipids of yeast, a subject which has not received much attention from earlier workers.

Hoppe-Seyler (2), in 1866, was the first to show that lecithin was present in yeast for he was able to isolate both choline and glycerophosphoric acid from the cleavage products of the yeast compound. The presence of cephalin in yeast was suggested by Koch (3) and proved by Austin (4). Investigations reported by Sedlmayr (5) and by Daubney and MacLean (6) indicate that palmitic and oleic acids are present as the fatty acid components.

The results of our own experiments confirm the conclusions of previous investigators that yeast contains both lecithin and cephalin, and, judging by the ratio of amino nitrogen to total nitrogen, the phospholipids we isolated consisted of a mixture of 4 parts of lecithin to 1 part of cephalin. From the water-soluble constit-

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uents, obtained on hydrolysis of the phospholipids, we were able to isolate both choline and aminoethyl alcohol as well as glycerophosphoric acid.

The ether-soluble constituents consisted of about 3 per cent of unsaponifiable matter and 66 per cent of fatty acids. The latter were composed of 14 per cent solid saturated acids and 86 per cent liquid acids. The saturated acids were composed of about equal parts of palmitic and stearic acids. The unsaturated liquid acids contained both C_{16} and C_{18} acids which on catalytic reduction were converted into palmitic and stearic acids. A very small amount of lauric acid was also present. Because of the low iodine number of the liquid acids, we believe that there were no acids present more highly unsaturated than oleic acid.

No attempt was made to separate the crude phospholipids into lecithin and cephalin, hence we do not know in which fraction the unsaturated C_{16} acid was present.

In the present investigation we searched in vain for compounds which are characteristic constituents of the phosphatides of acid-fast bacteria. Liquid saturated acids, such as tuberculostearic or phthioic acid (7), were not present and we were unable to find any carbohydrate in the water-soluble fraction (8).

<div style="text-align:center">EXPERIMENTAL</div>

Hydrolysis of Phospholipids—The purified phospholipids described in Paper 1 of this series (9) contained 4.22 per cent of phosphorus and 1.78 per cent of nitrogen. For the present investigation, 98.7 gm. of the substance were rubbed into a suspension with 1250 cc. of water which had been boiled, cooled, and saturated with carbon dioxide. About 55 cc. of concentrated sulfuric acid were added slowly and the mixture refluxed until the coagulum which had formed on acidification had been changed into a clear dark oil, the time required being 6 hours. The fatty acids were removed by extraction with ether and the aqueous solution was saved for the determination of the water-soluble compounds.

Unsaponifiable Matter—The crude fatty acids, obtained on evaporation of the ether, were refluxed with an excess of alcoholic potassium hydroxide. After dilution with water, the soap solution was extracted with ether. From this extract 2.96 gm. of unsaponifiable matter were obtained. The properties of this

substance indicated that it was identical with the mixture of saturated hydrocarbons contained in the acetone-soluble fat (9).

Separation of Fatty Acids—After isolation in the usual manner and drying in a vacuum desiccator, the fatty acids formed a dark oil weighing 65.3 gm. The iodine number (Hanus) was 79.5. The acids were separated by means of the lead soap-ether treatment (10) into 9 gm. of solid acids and 55 gm. of liquid acids having an iodine number of 84.9.

Liquid Fatty Acids—The liquid acids were reduced catalytically (11) after which the above lead soap-ether treatment was repeated. The solid reduced acids weighed 53.9 gm. while the ether-soluble portion of the lead salts yielded only 0.5 gm. of a semicrystalline mass. When we consider the very small amount of material thus obtained and also the fact that the mixed lead salts of palmitic and stearic acids have some slight solubility in ether, the conclusion must be drawn that yeast phospholipids do not contain any liquid saturated fatty acids similar to tuberculostearic or phthioic acid (7).

Examination of Reduced Fatty Acids—These acids were examined in the same way in which the various acid fractions of the acetone-soluble fat (9) were examined.

Three pure fractions were obtained and were shown to be methyl laurate, methyl palmitate, and methyl stearate by the following data.

Fraction I · M.p. 1 2°; $n_D^{25} = 1.4287$. The melting point of methyl laurate is 4-5° and $n_D^{25} = 1.4290$. The molecular weight of the ester as determined by saponification with an excess of alkali and titrating back with 0.1 N HCl was 215; calculated for methyl laurate, 214. The free acid melted at 42.5-43.5°. Lauric acid melts at 43-44°.

The silver salt was prepared and analyzed with the following result.

0.2787 gm. substance gave 0.0981 gm. Ag on ignition
$C_{12}H_{23}O_2Ag$ (306.88). Calculated, Ag 35.15; found, 35.20

Fraction II· M.p. 28-29°; $n_D^{55} = 1.4260$; free acid m.p. 62-63°.

0.4480 gm. substance required 17.44 cc. 0.1 N alcoholic KOH
$C_{16}H_{32}O_2$. Calculated mol. wt., 256; found, 256

Fraction III—M.p. 37.5–38.5°; $n_D^{55} = 1.4300$; free acid m.p. 70.5–71.5°.

> 0.5025 gm. substance required 17.62 cc. 0.1 N alcoholic KOH
> $C_{18}H_{36}O_2$.   Calculated mol. wt., 284; found, 285

From the quantities of the ester fractions and their indices of refraction we estimate that the reduced solid acids consisted of about 60 per cent palmitic and 40 per cent stearic acid.   The small amount of lauric acid obtained was not taken into consideration in these calculations.

Solid Saturated Fatty Acids—The solid saturated acids were methylated and the esters treated as above.   Two pure fractions were obtained and were shown to be methyl palmitate and methyl stearate, having the same physical properties as the corresponding esters mentioned under the reduced solid fatty acids.   The free acids had the correct melting points and showed no depression of the melting point when mixed with the corresponding authentic acid.   On titration correct values for the molecular weights were found.   For the sake of brevity these figures are not printed.   Approximately equal parts of palmitic and stearic acids were present in this fraction.

Distribution of Phosphorus and Nitrogen in the Hydrolysate—In a preliminary analysis in which 14.85 gm. of the phospholipids were hydrolyzed with dilute sulfuric acid, the aqueous solution remaining after the fatty acids had been extracted with ether was examined for phosphorus and nitrogen.   The sulfuric acid was removed quantitatively with barium hydroxide after which the filtrate was concentrated and made up to 100 cc.   Aliquots of this solution were analyzed and the following distribution of phosphorus and nitrogen was found.

	per cent
Total phosphorus (Neumann)	3.57
Phosphorus (inorganic H_3PO_4)..	. 0.31
Total nitrogen (Kjeldahl)...	1.59
Amino " (Van Slyke)..	. 0.53

The values for total phosphorus and nitrogen are somewhat lower than those found in the direct analyses of the phospholipids.   The difference is undoubtedly due to losses during the various manip-

ulations to which the solution had been subjected.   From the ratio
of amino nitrogen to total nitrogen, one can calculate the ratio of
aminoethanol to choline, which is about 1 to 4.

Identification of Water-Soluble Constituents

Isolation of Barium Glycerophosphate—The aqueous solution obtained on hydrolyzing the phospholipids was freed of sulfuric acid quantitatively with barium hydroxide. The resulting solution was neutralized with barium hydroxide and a precipitate which separated was filtered off, washed with water, and dried. This substance, which weighed 9.4 gm., consisted of barium phosphate and was discarded. The filtrate and washings were combined and the barium glycerophosphate was precipitated by adding an equal volume of alcohol. The precipitate was collected on a Buchner funnel and washed with alcohol. The filtrate was saved for the isolation of the nitrogen bases.

For purification the barium glycerophosphate was dissolved in water and reprecipitated by adding an equal volume of alcohol. This treatment was repeated until a snow-white precipitate was obtained. The purified product weighed 21.8 gm. For analysis it was dried to constant weight at 110° *in vacuo*.

0.7899 gm. substance gave 0.5513 gm. $BaSO_4$ and 0.2588 gm. $Mg_2P_2O_7$
$C_3H_7O_6PBa + 1.5H_2O$ (334.4).   Calculated.   Ba 41.09, P 9.27
                                          Found.   " 41.07, " 9.13

Isolation of Choline Chloride—The filtrate from the barium glycerophosphate precipitate was concentrated to a syrup under reduced pressure. This syrup was extracted with absolute alcohol and the alcoholic solution treated with an excess of mercuric chloride in alcohol. The precipitate was filtered off and washed with alcohol. The filtrate and washings were saved and examined as will be described later. The precipitate was suspended in water and decomposed with hydrogen sulfide. The mercury-free solution was concentrated to dryness *in vacuo* when stout transparent crystals were deposited. These were filtered off and washed with absolute ether. From the mother liquors a second crop was obtained. The two were united and dried, weighing 4.59 gm. For analysis, the

substance was dried to constant weight *in vacuo* over phosphorus pentoxide.

> 0.3867 gm. substance (Kjeldahl): 27.51 cc. 0.1 N HCl
> 0.2089 "   "   : 0.2135 gm. AgCl
> $C_5H_{14}ONCl$ (139.5).   Calculated.   N 10.04, Cl 25.42
>   Found.   " 9.95, " 25.28

Isolation of Choline Chloroplatinate—The filtrate from the choline chloride was evaporated to dryness, the residue was dissolved in alcohol, and an excess of an alcoholic solution of chloroplatinic acid added. The precipitate of choline chloroplatinate was filtered off, washed with cold alcohol, and dried to constant weight *in vacuo*. The yield was 8.11 gm. The filtrate was examined as will be described below.

The filtrate from the mercury chloride precipitate was freed from the excess mercury by treatment with hydrogen sulfide. The resulting solution was concentrated *in vacuo* to a thick syrup and the latter was dissolved in alcohol. The choline chloride present was removed by precipitation with chloroplatinic acid, 1.95 gm. being obtained. The filtrate was combined with the above mentioned filtrate and examined for aminoethyl alcohol. The choline chloroplatinate on recrystallization from dilute alcohol gave orange-colored crystals.

> *Analysis*—0.2663, 0.1253 gm. substance: 0.0835, 0.0393 gm. Pt
> $(C_5H_{14}ONCl)_2PtCl_4$ (616.2).   Calculated.   Pt 31.68
>   Found.   " 31.35, 31.36

Isolation of Aminoethanol Picrolonate—The combined filtrates from the choline chloroplatinate precipitates were concentrated to dryness and dissolved in water. The excess platinum was removed with hydrogen sulfide and the platinum-free solution was concentrated to dryness under reduced pressure and taken up in alcohol. A hot concentrated solution of picrolonic acid in alcohol was added and the mixture heated for a few minutes. As the solution cooled a heavy precipitate of fine yellow needles separated. The substance, after it had been recrystallized from alcohol, weighed 8.99 gm., melted with decomposition at 223-225°, and when mixed with authentic aminoethanol picrolonate the melting point was not depressed.

> *Analysis*—0.1791 gm. substance gave 33.5 cc. N_2 at 21° and 751 mm.
> $C_{12}H_{15}O_6N_5$ (325).   Calculated, N 21.53; found, 21.46

Anyone interested in further details of this work may consult the thesis by M. S. Newman on file in the Yale University Library.

In conclusion we acknowledge with pleasure our indebtedness to Dr. C. N. Frey of The Fleischmann Laboratories, New York, for supplying the yeast used in this investigation.

SUMMARY

The phospholipids of yeast were hydrolyzed and the cleavage products studied in detail.

Glycerophosphoric acid, choline, and aminoethanol were identified among the water-soluble constituents.

The saturated fatty acids consisted of about equal parts of palmitic and stearic acids.

The unsaturated acids on catalytic reduction yielded a mixture of about 60 per cent of palmitic acid and 40 per cent of stearic acid, together with a trace of lauric acid.

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STROPHANTHIN

XXVIII. FURTHER DEGRADATION OF STROPHANTHIDIN AND PERIPLOGENIN DERIVATIVES

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Conclusions as to the size and relative positions of three of the rings of strophanthidin and related aglucones, as well as to the positions of the unsaturated lactone side chain and hydroxyl groups, now rest on evidence obtained in a series of studies which we believe is quite conclusive. In the case of strophanthidin, this partial picture of the molecule is given in Formula I. Whether the lactone side chain is attached directly or through an additional carbon atom, as suggested in our study of the isomeric dihydrogitoxigenins,[1] is a question which has been under further investigation and will be left to a subsequent communication. The remaining problem of importance is the determination of the size and the position of Ring IV. In recent work a number of procedures has been studied. As a result of one of these, an additional carbon atom of Ring II (carbon atom (14)) appears now to be excluded as a point of conjugation of Ring II and Ring IV. This evidence has been obtained by the use of the method employed by Wieland Schlichting, and Jacobi[2] for the degradation of cholanic ester.

As starting material we have used the previously described[3] tribasic acid, $C_{21}H_{32}O_6$ (Formula II), which was obtained first as the anhydride by the hydrogenation of the unsaturated lactone anhydride, $C_{21}H_{24}O_5$, which had in turn been prepared by heating duodephanthondiacid with an acetic anhydride-acetyl chloride

[1] Jacobs, W. A., and Elderfield, R. C., *J. Biol. Chem.*, **100**, 671 (1933).

[2] Wieland, H., Schlichting, O., and Jacobi, R., *Z. physiol. Chem.*, **161**, 80 (1926).

[3] Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **92**, 323 (1931).

mixture.   The tribasic acid, for which we now propose the name *dephanthanic acid*, as the trimethyl ester yielded with phenyl magnesium bromide a neutral hexaphenyl triscarbinol (Formula III) as a solid, amorphous substance.   On oxidation with chromic acid degradation occurred with the formation of a substance which from the analysis and titration was found to be a dibasic acid of the formula $C_{17}H_{26}O_4$, *dephanthic acid*.   This was confirmed by the the formation of a *dimethyl ester*.   The same acid (and ester) was obtained through an analogous series of reactions in which methyl magnesium iodide was used.   The intermediate hexamethyl triscarbinol crystallized, but because of its great solubility was oxidized directly to the above dibasic acid without attempts at isolation.

It is obvious that the formation of such a dibasic acid involving loss of 4 carbon atoms could have resulted only from the oxidation on the one hand of the tetraphenyl or tetramethyl biscarbinol side chain with loss of three of the original carbon atoms of the dibasic acid side chain to a carboxyl group.   The loss of the remaining carbon atom must have been the result of the degradation of the third carboxyl group as the carbinol to the next lower acid. Therefore, the third carboxyl group must be attached to a CH_2 group.   The resulting substance, dephanthic acid, must therefore be a dibasic acid, $C_{17}H_{26}O_4$, of Formula IV.   If a ring (Ring IV) had been encountered at this point, a keto group would have resulted which on further oxidation with ring cleavage would give rise to a carbonyl and a carboxyl group or to two carboxyl groups. Thus, it can be concluded that carbon atom (14) of strophanthidin must be contained in a CH_2 group and that this carbon atom cannot be contained in Ring IV.

If we take for granted the validity of the assumed positions and sizes of Rings I, II, and III (Formula I) and that carbon atom (18) is contained in a CH_2 group,[4] only two positions can be considered for Ring IV, whether 5- or 6-membered, as given in the skeletal Formulas V and VI.

In a previous communication,[5] the formation of a hydrocarbon by dehydrogenation of strophanthidin with selenium has been de-

[4] Jacobs, W. A., and Elderfield, R. C., *J. Biol. Chem.*, **97**, 727 (1932); **99**, 693 (1932–33).

[5] Jacobs, W. A., and Fleck, E. E., *J. Biol. Chem.*, **97**, 57 (1932).

scribed, which is apparently a dimethyl phenanthrene. If the latter is a primary degradation product (and this can be only an assumption for the present), its formation cannot be conciliated with the above skeletal formulas.   The evidence relating to Rings I

I

, II

III

IV

V   VI
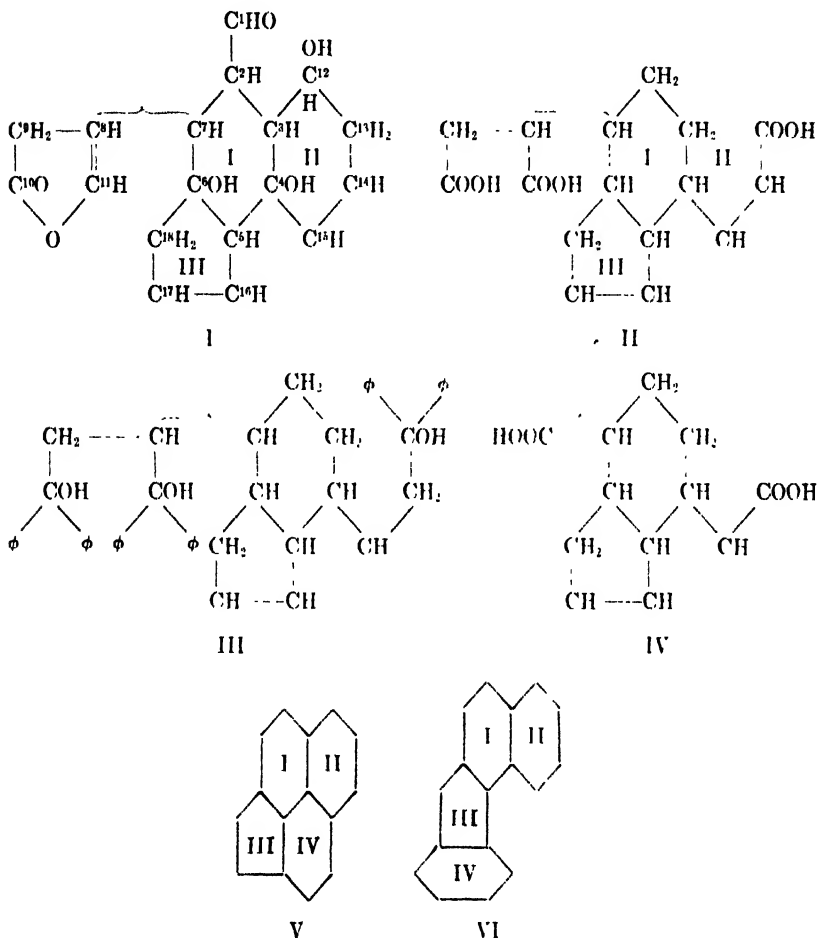
and II appears to be beyond question.   But there is a possible criticism which might be directed against the conclusions regarding Ring III; namely, that its position has been based on an assumption, which has often proved unreliable, of the position of the
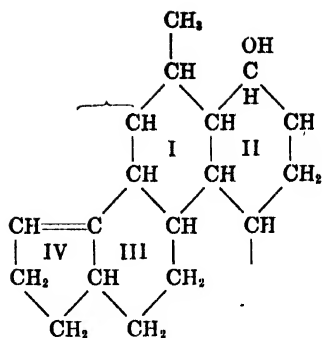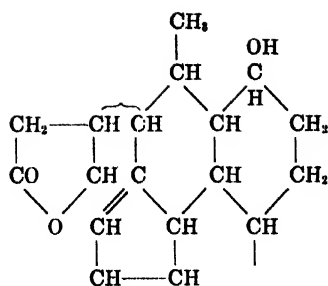
VII VIII

IX X

XI XII

XIII

240
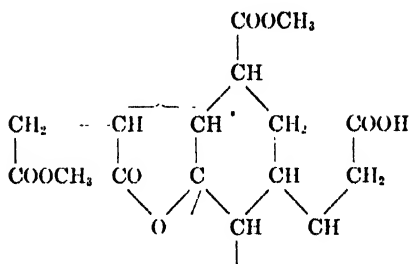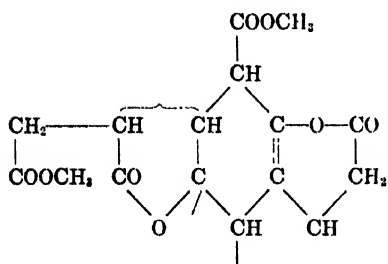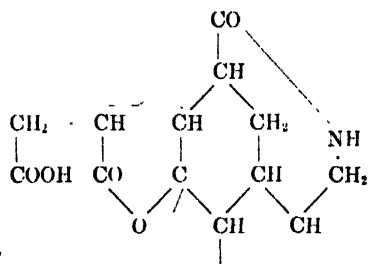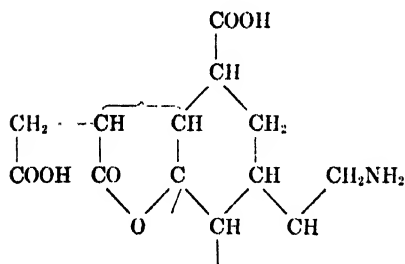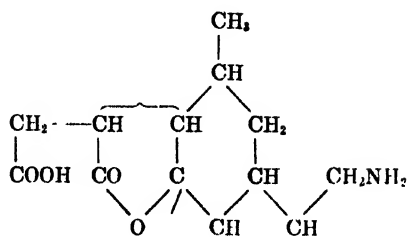
double bond formed on loss of hydroxyl[1] in dihydrostrophanthidin or dihydrodigitoxigenin, as shown in Formula VII.[4] In alkaline solution these unsaturated substances were oxidized to the dihydroxy derivatives which on still further oxidation gave δ-keto acids with cleavage of a 5-membered ring which was assumed to be Ring III, as in Formula VII.

By assuming a preliminary shift of this double bond to other positions, different conclusions might be reached. It is not inconceivable that in the alkaline solution such a shift might have occurred and to a fourth ring (Ring IV), as shown in Formula VIII, and that the 5-membered ring which was later cleaved could have been this Ring IV, and not Ring III. Under such circumstances, Ring III could thus still be 6-membered, and so the skeletal formula might be made to conform in general to the pattern recently derived for cholesterol and the bile acids. However, it would be difficult to make the relative positions which we have derived for the side chains of strophanthidin compatible with this conclusion.

There is no concrete evidence available to support any other than the original scheme which we have given in Formula I. But it is obvious that much more remains to be done to prove conclusively the positions and sizes of Rings III and IV, and further work is in progress with this end in view. In particular, as soon as sufficient of the costly and difficultly obtained dephanthic acid is procured its further degradation will be attempted

Simultaneously with the above degradation experiments, we have tried other procedures for the degradation of the molecule, especially the application of the Curtius method for the conversion of the carboxyl group to the next lower amine through the azide, or by means of the Hoffmann degradation of the amide. It was hoped to transform the amine to the alcohol which on oxidation would give either the corresponding acid or a carbonyl group. Unfortunately, this method of approach was finally discarded because of the difficulties encountered. Our experience was briefly as follows.

Because of its greater accessibility, undephanthontriacid dimethyl ester[3,6] was first used. On catalytic hydrogenation of the

[6] Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **79**, 539 (1928).

unsaturated lactone, $C_{24}H_{30}O_8$ (Formula IX), derived from this substance, a mixture was obtained of the corresponding *saturated neutral lactone*, $C_{24}H_{32}O_8$, and of the saturated desoxy acid, *undephanthantriacid dimethyl ester*, $C_{24}H_{34}O_8$ (Formula X).   The acid was converted through the *chloride* to *undephanthanamide dimethyl ester*.   This amide failed to yield a crystalline amine with hypobromite.   On the other hand, when the acid was put through the series, chloride, azide, urethane, the latter on decomposition with alkali did not give the desired amine (Formula XI) but a *lactam*, $C_{21}H_{29}O_5N$ (Formula XII), formed by condensation of the resulting amino group with the carboxyl group attached to Ring I.   The ready formation of this lactam group and its stability frustrated further efforts to use this substance for desamination to the hydroxy derivative.

. Since the carbomethoxyl group attached to Ring I was thus a disturbing factor, a similar series of reactions was attempted with the corresponding periplogenin derivative in which the carbomethoxyl group is replaced by an unreactive methyl group. Undeplogondiacid monomethyl ester, as described in a previous communication,[3] was converted into the unsaturated lactone, $C_{23}H_{30}O_6$.   The latter on hydrogenation gave only 25 per cent of the desired desoxy acid, *undeplogandiacid monomethyl ester*, $C_{23}H_{31}O_6$.   The major reaction product was a neutral substance, the investigation of which has not been completed.   When the desoxy acid was converted through the chloride into the azide, the latter on degradation gave only a very small yield of the amine (the aminolactone acid, $C_{21}H_{33}O_4N$) (Formula XIII) which was isolated as the ethyl ester hydrochloride.   However, the small yields thus obtained through a long series of steps starting from an aglucone which is so difficult and costly to procure as periplogenin appear to make further work with this substance almost hopeless.

A similar series of reactions was attempted beginning with both the tribasic acid, dephanthanic acid, and its anhydride but with unpromising results.   This procedure has therefore been discontinued in view of the greater promise of the previously discussed degradation of the Grignard product.

EXPERIMENTAL

Degradation of Dephanthanic Acid to Dephanthic Acid, $C_{17}H_{26}O_4$, by Means of the Grignard Reagent.   With Phenyl Magnesium

Bromide—A solution of 2.5 gm. of dephanthanic trimethyl ester, $C_{24}H_{38}O_6$, in 50 cc. of dry ether was slowly added to the Grignard reagent prepared from 1.98 gm. of magnesium and 9 cc. of bromobenzene in 300 cc. of dry ether. The mixture was refluxed for 6 hours and then allowed to stand overnight. After decomposition with ice and dilute sulfuric acid, the hexaphenyl triscarbinol was extracted with ether. After concentration the residue was subjected to steam distillation for the removal of diphenyl, etc. The non-volatile material which remained was again extracted with ether. After removal of solvent, the residue was boiled for 2 hours with 500 cc. of 0.5 N alcoholic KOH for removal of any incompletely reacted ester. The alcoholic solution was concentrated to about 100 cc., diluted, and then extracted with ether. After concentration, the crude hexaphenyl triscarbinol solidified to amorphous flocks under methyl alcohol and was collected. It was oxidized as such.

2.5 gm. of the hexaphenyl compound were dissolved in 100 cc. of acetic acid and 5 gm. of CrO_3 dissolved in 5 cc. of water were added. The mixture was heated on the steam bath for 7 hours and then concentrated under diminished pressure to about 20 cc. After dilution it was extracted with ether. The latter was first washed free from acetic acid with water, and then was extracted with dilute Na_2CO_3 solution. After acidification of the carbonate solution the acid slowly crystallized as leaflets. This was recrystallized from a small volume of strongly chilled dry acetone and then melted at 242-243° with softening above 200°. The yield was 0.2 gm. The substance is somewhat sparingly soluble in dry acetone but easily soluble in moist acetone. It possesses practically no optical activity. It appeared to be contaminated with a small amount of a persistent impurity which caused a slight discrepancy in the analytical figures, but this was corrected on conversion to the dimethyl ester given below.

$C_{17}H_{26}O_4$. Calculated. C 69.34, H 8.91
Found.   " 69.78, " 8.66
" 69.74, " 8.71

14.365 mg. of substance were titrated directly against phenolphthalein with 0.1 N NaOH. Calculated for 2 equivalents, 0.976 cc.; found, 0.919 cc.

The neutral part of the reaction product consisted largely of

benzophenone together with a small amount of non-crystalline material.

With Methyl Magnesium Iodide—The same acid was obtained by the interaction of the trimethyl ester and methyl Grignard reagent followed by subsequent oxidation as described above.   It melted at 243–245° after softening above 200°.

Found.   C 69.77, H 8.84

Dephanthic Dimethyl Ester—The ester was prepared from the above acid from either source with diazomethane.   It is very soluble in all of the usual solvents and after recrystallization from dilute acetone forms leaflets which melt at 69-71°.

$C_{19}H_{30}O_4$.   Calculated.   C 70.76, H 9.32, OCH_3 19.31
          Found.   " 70.94, " 9.27
                          " 70.70, " 9.16,   "   18.64

13.720 mg. of substance were refluxed with 2 cc. of alcohol and 3 cc. of 0.1 N NaOH for 4 hours and then titrated back against phenolphthalein.   Calculated for 2 equivalents, 0.852 cc.; found, 0.873 cc.

Hydrogenation of Unsaturated Lactone from Undephanthontriacid Dimethyl Ester to Undephanthantriacid Dimethyl Ester and the Saturated Lactone, $C_{24}H_{32}O_8$—5 gm. of the unsaturated lactone dissolved in acetic acid were shaken with 0.5 gm. of Adams and Shriner's catalyst in an atmosphere of hydrogen.   After several hours approximately 2 mols of hydrogen had been absorbed.   The filtrate from the catalyst was concentrated to dryness *in vacuo* and the residue was dissolved in chloroform.   The chloroform solution was extracted with dilute Na_2CO_3 solution.   On acidification of the carbonate extract with HCl the desoxy acid, *undephanthantriacid dimethyl ester*, crystallized as leaflets.   After recrystallization from dilute acetone it melted at 201°.   It is readily soluble in the usual solvents except ether and petrolic ether.   The yield was 2.7 gm.

$C_{24}H_{34}O_8$.   Calculated.   C 63.96, H 7.61
          Found.   " 64.12, " 7.84

The chloroform solution of neutral material consisting of the saturated lactone was concentrated to dryness.   The residue

readily crystallized from acetone as square plates and melted at 225–227°.

$C_{24}H_{32}O_8$.   Calculated.   C 64.25, H 7.19
        Found.   " 64.10, " 7.09

Undephanthanamide Dimethyl Ester, $C_{24}H_{35}O_7N$—0.5 gm. of the above desoxy acid, $C_{24}H_{34}O_8$, was covered with 5 cc. of thionyl chloride and allowed to stand at room temperature for 30 minutes. The excess thionyl chloride was removed at low pressure and the residue was taken up in dry chloroform.   Dry ammonia gas was passed through the solution until in excess.   After standing 1 hour, the precipitate was collected and recrystallized by careful dilution of its acetone solution.   The amide forms prisms which soften at 110–111° and melt sharply at 205°.

$C_{24}H_{35}O_7N$.   Calculated, N 3.12; found, N 2.93

Attempts to use this substance for degradation to the amine by Hoffmann's method were unsuccessful.

Lactam, $C_{21}H_{29}O_5N$, from Undephanthantriacid Dimethyl Ester— 2 gm. of the above desoxy acid were converted into the acid chloride by means of thionyl chloride as in the case of the amide.   After removal of the excess reagent, the residue was repeatedly concentrated with dry benzene to remove the liberated HCl.   The acid chloride crystallized as prisms during this treatment.   It was dissolved in 40 cc. of dry benzene and 3 gm. of freshly prepared sodium azide were added to the solution.   After standing at 40° for 4 hours, during which a slow but steady gas evolution occurred, the mixture was refluxed for 5 hours during which moisture was carefully excluded.   About 30 cc. of benzene were then distilled off and 40 cc. of absolute methyl alcohol were added.   The mixture was then refluxed a further 5 hours.   After dilution the resulting urethane was extracted with chloroform.   After removal of the chloroform the crude urethane was heated for 16 hours in a sealed tube at 100° with 30 cc. of 25 per cent alcoholic KOH solution. The diluted reaction mixture was acidified to Congo red with HCl and then treated with ammonium sulfate to about half saturation. After standing overnight, the partly crystalline lactam was collected with water.   The substance was recrystallized from a very

large volume of acetone in which it is very sparingly soluble. It forms fine needles which are very sparingly soluble in the usual solvents. The lactam melts at 264–266°.

$C_{21}H_{29}O_5N$. Calculated. C 67.16, H 7.78, N 3.73
Found. " 67.53, " 7.92, " 3.94

The presence of one carboxyl group and one lactone group was shown by the following titration.

12.205 mg. of substance were titrated directly with 0.1 N NaOH against phenolphthalein. Calculated for 1 equivalent, 0.325 cc.; found, 0.334 cc. 3 cc. of 0.1 N NaOH were then added, and after refluxing for 4 hours the solution was titrated back. Found, 0.342 cc.

Methyl Ester of Lactam Acid, $C_{21}H_{29}O_5N$—The above lactam acid was esterified in acetone suspension with diazomethane. The lactam ester formed fine prisms from acetone in which it is sparingly soluble. It melts at 226–228°.

$C_{22}H_{31}O_5N$. Calculated. C 67.81, H 8.04
Found. " 68.17, " 8.21

Hydrogenation of Unsaturated Lactone from Undeplogondiacid Monomethyl Ester to Undeplogandiacid Monomethyl Ester—1.1 gm. of the unsaturated lactone, $C_{23}H_{30}O_6$,[3] were hydrogenated in acetic acid solution with the catalyst of Adams and Shriner. After several hours absorption stopped at slightly less than 2 mols of hydrogen. The filtrate from the catalyst was concentrated to dryness at low pressure. The residue was dissolved in chloroform and the solution was extracted with dilute Na_2CO_3 solution. The carbonate extract on acidification with acetic acid deposited fine prisms of the desoxy acid. After recrystallization from dilute acetone, the substance formed hexagonal plates and melted at 212°. The yield was 0.3 gm.

$C_{23}H_{34}O_6$ Calculated. C 67.94, H 8.43
Found. " 68.27, " 8.51

The chloroform solution containing the neutral reaction product was dried and concentrated. After repeated recrystallization from acetone it formed rectangular prisms which melted at 189–190° after slight preliminary softening. However, the analytical figures

obtained did not conform with those of the expected saturated lactone.   Investigation of the cause of this discrepancy has been left for the present.

$$C_{22}H_{12}O_6.\quad \text{Calculated.}\quad \text{C 68.27, H 7.98}$$
$$\text{Found.}\qquad \text{“ 69.80, “ 8.67}$$
$$\text{“ 70.13, “ 8.55}$$

Aminolactone Ester from Undeplogandiacid Monomethyl Ester— 0.4 gm. of the desoxy acid was converted into the chloride with thionyl chloride.   After removal of the excess reagent and HCl by repeated concentration with dry benzene, the chloride was refluxed in 25 cc. of dry benzene with 1 gm. of freshly prepared sodium azide for 5 hours.   20 cc. of benzene were then distilled off. After addition of 25 cc. of absolute methyl alcohol the mixture was refluxed for 3 hours.   During these operations moisture was rigidly excluded.   After dilution the urethane was extracted with chloroform.   The chloroform solution was concentrated to dryness and the residue was heated for 16 hours at 100° with 10 cc. of 25 per cent alcoholic KOH solution.   The urethane was thus cleaved to the amine and simultaneously the ester and lactone groups were saponified.   The contents of the tube were made just acid to Congo red with HCl and then concentrated to dryness. The residue, consisting of the hydrochloride of the aminolactone acid mixed with inorganic salt, was repeatedly concentrated with absolute ethyl alcohol to remove water.   Extraction of the residue with absolute alcohol readily removed the amine hydrochloride from the insoluble inorganic salts.   Dry hydrogen chloride was passed into the alcoholic solution until it contained about 2 per cent, and the mixture was allowed to stand overnight to esterify the free carboxyl group.   The solution was then made neutral to Congo red by addition of sodium acetate and then concentrated to dryness. After dissolving in water, the solution was made alkaline with Na_2CO_3 and extracted with ether.   Thus, separation from impurities was insured.   The ether extract yielded a residue which was repeatedly boiled down with benzene in order to remove all moisture.   Finally, it was taken up in absolute ether and treated with dry hydrogen chloride in excess.   An immediate flocculent precipitate formed which crystallized as leaflets on longer standing.   The substance was collected with dry ether.   It is somewhat

hygroscopic and very soluble in the usual solvents except ether and petrolic ether.   It melts slowly at 100–120°.   For analysis it was dried at 80° and 15 mm.

$C_{23}H_{37}O_4N \cdot HCl$.   Calculated.   C 64.52, H 8.95, N 3.27
                          Found.   " 65.09, " 8.93, " 3.55 (Dumas)

On shaking 15 minutes in the Van Slyke apparatus only 2.08 per cent of amino nitrogen was evolved, and after shaking for 1 hour but 2.49 per cent.

Attempts to crystallize the free amino ester were unsuccessful.

THE PREPARATION OF GLYCYLTAURINE AND GLYCYL-CYSTEIC ACID*

By JULIUS WHITE†

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Although a chemical relationship between cystine and taurine has been demonstrated (1–3), uncertainty exists as to the biological relationship between cystine and taurine or taurochloric acid (4–10). With the purpose of increasing our knowledge of the reactions of cysteic acid and taurine, the synthesis of peptides containing these sulfur compounds has been undertaken.

EXPERIMENTAL

Glycyltaurine—The method used by Abderhalden and Riesz (11) for the preparation of leucyltaurine was employed. 6.25 gm. (0.05 mol) of pure taurine (synthetic) were placed in a 250 cc. Erlenmeyer flask and dissolved in 70 cc. (0.07 mol) of N sodium hydroxide. After all the taurine was in solution, the flask was cooled in an ice bath for 30 minutes. To this were added alternately in five portions, 15.15 gm. (0.075 mol) of bromoacetylbromide and 150 cc. of N sodium hydroxide. After each addition the mixture was allowed to stand 1 hour in the ice bath and was then made acid to litmus with 5 N hydrochloric acid. Attempts to extract the product from the aqueous solution with either ethyl acetate, ether, or chloroform failed. The aqueous solution was evaporated to dryness on a steam bath under reduced pressure. The residue resulting was dissolved in 125 cc. of concentrated ammonium hydroxide (sp. gr. 0.90). This was allowed to stand 5 days at

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room temperature.   The excess ammonia and water were then removed on a steam bath under reduced pressure.   The residue remaining was dissolved in 50 cc. of hot water and 250 cc. of alcohol were added.   On cooling the solution in an ice bath, glycyltaurine separated out, which on recrystallization from a minimum amount of hot water, was obtained in rectangular platelets.   The yield was 4.5 gm. (50 per cent of theoretical).

Analysis
0.1505 gm. substance: 16.3 cc. of 0.1 N HCl (Kjeldahl)
0.1031   "   "   : 0.1328 gm. BaSO₄
$C_4H_{10}O_4N_2S$.   Calculated.   N 15.38, S 17.58
Found.   " 15.16, " 17.68
Estimation of NH₂-N (12)
Calculated.   0.1475 gm. required 8.11 cc. 0.1 N HCl
Found.   0.1475 "   "   8.17 " 0.1 "   "
Estimation of COOH and other acidic groups (12)
Calculated.   0.1475 gm. required 8.11 cc. 0.1 N NaOH
Found.   0.1475 "   "   8.05 " 0.1 "   "

Glycylcysteic Acid—Glycylcysteic acid could not be prepared in a manner analogous to that used for glycyltaurine.   The bromoacetylbromide does not condense with cysteic acid.   This is not surprising because the oxidation of the sulfur of cystine to a sulfonic acid group results in the accumulation of negative groups which in turn should decrease the reactivity or basicity of the amino group.

7.08 gm. of diglycylcystine (prepared by the method of Abderhalden and Spinner (13)) were dissolved in 200 cc. of water.   To this were added dropwise, 16 gm. of bromine with continuous stirring and cooling in the ice bath.   After all the bromine was added the solution was allowed to stand at room temperature for 30 minutes.   The solution was then evaporated on a steam bath under reduced pressure to a syrup.   The syrup was rubbed up in absolute alcohol.   After several rubbings the product began to crystallize.   The rubbing was continued until the test for the bromide ion was negative.   The yield was 8.5 gm. (91 per cent of theoretical).

Analysis
0.1548 gm. substance: 13.8 cc. 0.1 N HCl (Kjeldahl)
0.1374   "   "   : 0.1425 gm. BaSO₄
$C_5H_{10}O_5N_2S$.   Calculated.   N 12.38, S 14.15
Found.   " 12.44, " 14.24

Estimation of NH_2-N

      Calculated. 0.1060 gm. required 4.69 cc. 0.1 N HCl
      Found.   0.1060 "   "   4.70 " 0.1 " "

Estimation of COOH and other acidic groups

      Calculated. 0.1060 gm. required 9.38 cc. 0.1 N NaOH
      Found.   0.1060 "   "   9.45 " 0.1 "   "

The behavior of peptides of this type is of particular interest since in the bile acids containing taurine, the amino group of the taurine is combined and is not liberated until after hydrolysis. A biological study of these new peptides is now in progress.

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THE DECOMPOSITION OF CYSTINE PHENYLHYDANTOIN*

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The series of investigations carried out by Bergmann and co-workers on the possible mechanisms for the decomposition of amino acids has emphasized the great instability of certain cyclic derivatives of such amino acids as serine, cystine, and cysteine when in the presence of small amounts of alkali (1). Studies have already been published on the properties and mode of decomposition of diketopiperazines and azlactones and Bergmann and Delis (2) have reported studies on the mode of decomposition of serine phenylhydantoin. These cyclic compounds of serine in the presence of small amounts of alkali easily lose 1 molecule of water at the hydroxy group and give a methylene derivative, whereas, in the case of cystine derivatives, the corresponding reaction produces H_2S and free sulfur. In the present paper we wish to report the results of a series of studies on the properties and mode of decomposition of cystine phenylhydantoin. In general, it may be stated that the mechanism of the decomposition of this compound in alkaline solution corresponds very closely to that observed by Bergmann and Delis for the corresponding serine compound.

EXPERIMENTAL

Physical Properties—Cystine phenylhydantoin was prepared from *l*-cystine by a procedure essentially the same as that de-

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scribed by Patten (3). It was generally found necessary, how-
ever, to boil the hydantoic acid longer with hydrochloric acid
(20 per cent) than is described by Patten in order to form the
hydantoin. This procedure usually gives yields of from 50 to 60
per cent of the theory. The product had most of the solubilities
mentioned by Shiple and Sherwin (4). It is also very soluble in
pyridine. Contrary to the statement of Shiple and Sherwin, it is
moderately soluble in both hot water and hot HCl and also
(with decomposition) in dilute alkalies. The molar solubilities
of *l*-cystine phenylhydantoin in water and 1:1 hydrochloric acid
are as follows:

Temperature	H₂O	1 : 1 HCl
°C.	*mols per l* × 10⁴	*mols per l* × 10⁴
28	1 4	5 7
38	2 4	7 0
60	10 1	16 8
100	55 4	65 5

The rapidly mounting solubility indicates why the hydantoin
can be crystallized from the boiling hydrochloric acid in which the
hydantoic acid is heated. These solubilities were obtained from
Kjeldahl determinations on large samples of the saturated solu-
tion. The solutions were filtered through hardened filter paper
at the temperature of the determination. The figures for water,
especially at 60° and 100°, are less certain than those for hydro-
chloric acid, since slow decomposition proceeds in water and the
product (methylene phenylhydantoin) is more soluble. This
decomposition appears to be largely inhibited in 1:1 hydrochloric
acid. The figures at 100° were obtained by adding varying
amounts of water or acid at 100° to weighed samples until solu-
tion was just effected. They are obviously approximations but
illustrate the rapid increase in solubility with rise in temperature.
In boiling water the odor of hydrogen sulfide is very evident.
The hydantoin crystallizes satisfactorily from either 95 per cent
alcohol, glacial acetic acid, or acetone.

The optical activity varies, depending on the activity of the
original cystine, but for samples made from a good grade of
l-cystine ($[\alpha]_D^{25}$ greater than $-210°$), $[\alpha]_D^{25}$ in anhydrous acetone

$= -155°$. The degree of exposure to boiling hydrochloric acid during preparation also appears to influence the proportion of optical isomers in the product. In anhydrous pyridine the optical activity averages about 63 per cent higher. Thus, an $[\alpha]_D^{25}$ value of $-155°$ in acetone corresponds to $-253°$ in pyridine. These last two figures are those most commonly obtained from cystine phenylhydantoin made from pure *l*-cystine. On one such sample, four successive recrystallizations from anhydrous acetone gave in each case crystals of $[\alpha]_D^{25}$ (acetone) $= -155°$ and melting at 114.5°.

This melting point is somewhat lower than that of previous investigators, particularly of Gortner and Hoffman (5) whose value 122 123° may represent slight contamination with the racemic form. These authors report 166° for the melting point of cystine phenylhydantoin from racemic cystine. For this material we have obtained 177°. It should be noted, however, that small amounts of an amorphous phase are very easily formed in the crystallization of this material and are very effective in producing ambiguous melting points. This is particularly true of the racemic form. Although a rough relation exists between melting point and optical activity values of cystine phenylhydantoin samples, this relation is rendered very irregular by these melting point variations.

Mode of Decomposition—On the basis of the mode of decomposition previously established by Bergmann and coworkers for cystine dialanine dianhydride and for serine phenylhydantoin the following reactions would be expected for cystine phenylhydantoin (6).

$$+ H_2S + S$$
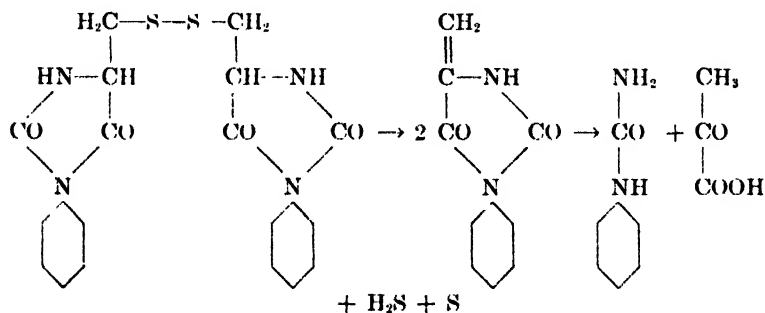
The first step, involving the formation of 3-phenyl-5-methylene hydantoin and consequent loss of the asymmetric carbon atom, is most easily followed in the polarimeter. The samples were dissolved in either acetone or pyridine, the solutions in some cases containing in addition some water. Tables I and II show the results of a number of such measurements for acetone and pyridine solutions respectively.

From these data certain conclusions may be drawn: Cystine phenylhydantoin is quite stable at room temperature in acetone solution, both anhydrous and aqueous, and is moderately stable in anhydrous pyridine. In aqueous pyridine solution, the

TABLE I

Rate of Loss of Optical Activity of 1 Per Cent Solutions of Cystine Phenylhydantoin in Various Acetone-Water Solutions at 21°

Time	Pure anhydrous acetone, no NaOH	75 per cent acetone, no NaOH	80 per cent acetone, 1:1 M hydantoin to NaOH	80 per cent acetone, 4:1 M hydantoin to NaOH	80 per cent acetone, 10:1 M hydantoin to NaOH	80 per cent acetone, 15:1 M hydantoin to NaOH	80 per cent acetone + excess HCl
min	degrees	degrees	degrees	degrees	degrees	degrees	degrees
0	−155	−123	−120	−124	−125	−128	−102
10	−154		−75	−121	−114	−126	
20	−153		−38	−118	−113	−122	
30		−123	−20	−116	−112	−118	
50	−154		−4	−113	−111	−117	−102
80	−153			−104	−109		
120		−123	0	−96	−107	−116	−102
300		−123		−71	−103	−114	−102
hrs.							
24	−153	−122		−54			

alkalinity of the solvent produces a decomposition comparable in speed with that observed when small amounts of alkali have been added. With either solvent a 1:1 M ratio of hydantoin to alkali causes nearly complete decomposition in an hour. With very small amounts of alkali (Table I, next to last column) the rate of decomposition, after reaching zero, may be again accelerated by a second addition of the original amount of alkali, the amount first added having been neutralized by the hydrogen sulfide resulting from the decomposition. In Table I, only the effect of the first addition of alkali is shown.

The effect on the initial optical activity of the introduction of water into the anhydrous solvent is noteworthy. Other measurements of optical activity of the hydantoin in acetone and pyridine which were not carefully dehydrated emphasized the pronounced effect on the optical activity of small amounts of water.

The values for zero time were extrapolated—a process of obviously limited value with the more rapidly changing solutions.

The rate of decomposition in the dilute alkali is also influenced catalytically by contact with glass. The data in Tables I and II

TABLE II

Rate of Loss of Optical Activity of 1 Per Cent Solutions of Cystine Phenyl-hydantoin in Various Pyridine-Water Solutions at 21°

Time	Pure anhydrous pyridine, no NaOH	50 per cent pyridine, no NaOH	50 per cent pyridine, 1.1 M hydantoin to NaOH	80 per cent pyridine, 4.6 1 M hydantoin to NaOH	80 per cent pyridine, 25 1 M hydantoin to NaOH	80 per cent pyridine, 1.1 M hydantoin to HCl	80 per cent pyridine + excess HCl
min.	*degrees*	*degrees*	*degrees*	*degrees*	*degrees*	*degrees*	*degrees*
0	-251	-112	-100	-114	-127	-131	-94
10	-249	-107	-58				
20	-246	-101	-29	-118		-131	
30	-245	-98	-16	-113	-127		-93
60	-242	-86	-3	-100	-122		-93
120		-71	0	-97	-113		
160	-231					-127	
300	-220			-82	-107		-75
400	-214				-101	-113	
hrs							
24	-203	-21			-87	-76	-51

all apply to solutions in glass flasks but it was demonstrated that in paraffin-lined containers the rate was much decreased. In acid, the hydantoin appears to be much more stable. The slow loss of activity at room temperature appears to be merely a racemization, since we have never succeeded in isolating any of the decomposition products from acid solutions which had partially lost their optical activity under such conditions.

Attempts to calculate definite velocity constants from the data from which Tables I and II are abbreviated gave values which were not constant for either a first or a second order reaction but

which indicated a mixture of the two.   This is probably a result of simultaneous decomposition and racemization.

The decomposition of cystine phenylhydantoin was also followed in water and in hydrochloric acid suspensions at 100° by measurement of the hydrogen sulfide evolved.   This was swept into ammoniacal cadmium chloride solution and the cadmium sulfide was filtered, washed, and decomposed with hydrochloric acid in the presence of excess standard iodine.   The results (Table III) are expressed in terms of percentage of the original hydantoin decomposed.   The greater stability of the compound in strongly acid solution is again emphasized, although the form of the curves produced by these data indicates less difference between water and acid at lower percentages of decomposition.   An

TABLE III

*Decomposition of Cystine Phenylhydantoin Suspensions in Water and in 1:1
HCl As Measured by H_2S Production (at 100°)*

	Decomposition after			
	3 hrs	6 hrs	12 hrs	18 hrs
	per cent	per cent	per cent	per cent
Water suspension	20 1	40 6	72 0	98 6
1:1 HCl "	18 0	27 5	46 1	61 5

increasing degree of negative catalysis in hydrochloric acid would seem to be indicated.

In the presence of excess lead the reaction is much accelerated. At 100°, with lead acetate added directly to the water suspension and the decomposition measured by weighing the resulting lead sulfide, the percentage decomposition varied from 30 per cent in 10 minutes to 79 per cent in 40 minutes.   Attempts to use the precipitation of lead sulfide as an analytical method for the determination of cystine phenylhydantoin have failed.   The yield of lead sulfide obtained from pure samples varied from about 75 per cent of the theory to higher values, the latter doubtless resulting from further interaction of the free sulfur with the alkali.

Attempts made to determine whether any side reactions resulting in production of ammonia took place gave completely

negative results.   A 1 per cent solution of the hydantoin in 4.0 N NaOH produced no ammonia in 6 days at room temperature.

Identification of Decomposition Products—The products of decomposition of cystine phenylhydantoin as indicated by the above equation have been isolated.   3-Phenyl-5-methylene hydantoin was obtained in the form of prisms with the properties described by Bergmann and Delis with a nitrogen content of 14.53 per cent (theory 14.90).   Because of its further instability in dilute alkali, this compound is not easily prepared pure.   The further split to pyruvic acid and phenylurea proceeds with great ease.

Phenylurea was obtained by dissolving cystine phenylhydantoin in 4 N NaOH at room temperature and adding an equal volume of 50 per cent NaOH, in which phenylurea is comparatively insoluble.   This procedure gives a yield of 85 per cent of the theoretical.   The phenylurea was obtained in the usual form of large flat prisms, melting at 146° and with a nitrogen content of 20.50 per cent (theory 20.58).

The presence of pyruvic acid was demonstrated in the filtrates from phenylurea preparations such as the above by precipitation of pyruvic acid phenylhydrazone as described by Bergmann and Delis (6) and by Clarke and Inouye (7).   Our product melted at 191°, in good agreement with the results of the above authors. The preparation of pyruvic acid *p*-carboxyphenylhydrazone as described by Clarke and Inouye was also carried out.   The product had the properties described by these authors but the melting point was, because of decomposition, which they also report, a somewhat indefinite figure between 252–258°.

Racemic Cystine Phenylhydantoin—By use of inactive cystine, racemic cystine phenylhydantoin was prepared by the same procedure as that used for the levo compound.   This compound was obtained in the crystal form described by Gortner and Hoffman (5).   Our melting point, obtained on a twice recrystallized sample, was 177° (Gortner and Hoffman report 166°), but since this compound assumes the amorphous condition even more easily than does the *l* form, these values are subject to wide variations.   Racemic cystine phenylhydantoin is several times more soluble in both acetone and water than is the *l* form.   Its mode of decomposition is the same.

Oxidation of Cystine Phenylhydantoin—Because of the possibility of the formation of taurine from the decomposition of the corresponding phenylhydantoin of cysteic acid, the production of this compound by direct oxidation of cystine phenylhydantoin was investigated.   This method was resorted to since preliminary attempts to add the phenylhydantoin ring directly to cysteic acid were unsuccessful.   The oxidation was accomplished by grinding a paste of the cystine phenylhydantoin under bromine water at 0° until a qualitative test with sodium plumbite showed no lead sulfide.   The crude cysteic acid phenylhydantoin is best recrystallized by dissolving in cold acetone (9 to 10 gm. per 100 cc. of acetone), filtering, and adding an equal volume of cold water *as rapidly as possible.*

$$H_2C\!-\!SO_3H$$

$$CH\!-\!NH$$

$$CO\quad N$$
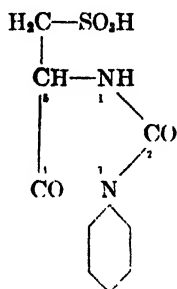
The compound crystallizes out at once as the hydrobromide in silvery fragile plates.   Delay in reprecipitating results, even at room temperature, in complete decomposition and loss of the compound.   As described above, the reprecipitation rarely yields more than 50 per cent of the original weight of crude cysteic acid phenylhydantoin hydrobromide.

The compound appears not to have been previously described in the literature.   It melts sharply at 138° and is insoluble in ether, amyl alcohol, and *n*-butyl alcohol.   It is moderately soluble in chloroform and absolute ethyl alcohol and very soluble in methyl alcohol, acetone, glacial acetic acid, and pyridine.   The great instability of the compound, however, makes it very probable that most of the above positive solubility tests are due to rapid decomposition, since only from acetone has it been possible to recover the unchanged compound (see above).   With pyridine

dark colored decomposition products are at once formed. $[\alpha]_D^{27} =$ $-41.4°$ in absolute acetone.

The recrystallized compound gives the following analysis.

	Found	per cent	Theory for $C_{10}H_{11}N_2O_5SBr$ (mol. wt. 351.13) per cent
N		7 96 (Kjeldahl)	7.98
S		9 12 (Benedict)	9.12
Br..		22.43 (Titration)	22 76

That the bromine is present as hydrobromide rather than on the ring is demonstrated by its quantitative titration directly by standard silver nitrate. Best results are obtained by adding excess standard silver nitrate solution directly to the dry compound and heating to boiling. Immediate decomposition ensues with precipitation of silver bromide, after which the back titration may be carried out in the usual way with thiocyanate. It appears necessary to assume that the HBr is attached to the NH group (position (1)).

Direct titration in 50 per cent acetone-water solution with 0.1 N NaOH, with either methyl red or alizarin as indicator, gave equivalent weights varying from 166 to 174. Assuming titration of both HBr and the sulfonic acid group, the theoretical equivalent should be $351.13 \div 2 = 175.6$. However, this end-point quickly faded towards the acid side, requiring constant addition of more alkali and became permanent only when approximately a 3rd equivalent of NaOH had been added. Addition to the sample of more than 3 equivalents of NaOH and back titration of the excess to a methyl red end-point gave an equivalent weight of 117.3 (theory $351.13 \div 3 = 117.04$). It is obvious that the rapid decomposition liberates a third acid group of such strength as to be quantitatively titrated at about pH 5. The simplest assumption to account for this third acid group is that the ring splits between positions (3) and (4), forming a corresponding hydantoic acid. It should be noted, however, that this implies a surprisingly low pK value for a carboxyl group. Furthermore, preliminary experiments have demonstrated that this compound easily forms considerable inorganic sulfate as one of its decomposition products, a fact which sharply distinguishes it from cysteic

acid and other sulfonic acids and which is possibly of significance in the mechanism of sulfur oxidation.

Neither the hydantoin nor the hydantoic acid gives evidence of any free amino nitrogen by the Van Slyke determination.   It has not yet been found possible to crystallize the free hydantoin.

Detailed studies of the mode of decomposition of this compound are now in progress.

SUMMARY

The mode of decomposition of cystine phenylhydantoin in both acid and alkali has been studied and found to correspond to that of serine phenylhydantoin.

Alkaline decomposition of cystine phenylhydantoin produces sodium sulfide, free sulfur, and 3-phenyl-5-methylene hydantoin, the latter easily decomposing to form pyruvic acid and phenylurea.

Mild alkaline decomposition at room temperature produces no ammonia.

The solubility of cystine phenylhydantoin in water and in hydrochloric acid at various temperatures has been determined.

The preparation and some properties of cysteic acid phenylhydantoin hydrobromide are described.   The compound is very unstable and gives, as one of its decomposition products, inorganic sulfate.

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THE RACEMIZATION AND OXIDATION OF CYSTINE IN ACID SOLUTION*

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In a previous paper (1) the writer published the results of some rather protracted experiments in which the oxidation of cystine to cysteic acid by means of atmospheric oxygen was demonstrated. In the present paper are reported the results of a further study of the mechanism of this oxidation. It has been found possible to accelerate catalytically the absorption of oxygen by the cystine to the point of accomplishing a complete conversion of several gm. of cystine to cysteic acid in about 10 days. The various factors involved are described below.

EXPERIMENTAL

Racemizaton of Cystine—Further studies of the rate of racemization of cystine in acid at 38° and 60° have confirmed the fact that the curve of optical activity against time is free from the distortion produced by oxidation of the *l*-cystine only if the solutions are carefully protected from oxygen or if the use of hydrochloric acid as a solvent is avoided. On the other hand, specific catalytic effects of different solvent acids on the rate of racemization are not lacking. Although this rate as indicated by velocity constants for 2.5 N HCl and H_2SO_4 is in each case about 1.2×10^{-4} at 38°, the corresponding constant in 2.5 M H_3PO_4 is 7.1×10^{-4}. Since the second and third dissociation constants of H_3PO_4 can hardly be regarded as having any appreciable effect under these conditions, the marked increase in speed of racemization must be attributed to the specific effect of the phosphoric acid.

* An abstract of this paper was published in *Proc. Am. Soc. Biol. Chem.*, **8**, iv (1933); *J. Biol. Chem.*, **100**, iv (1933).

Oxidation of Cystine—Tables I and II record the results of experiments at 38° and 60° respectively in which both racemization and oxidation played a part. In each series, the cystine solution in question was placed in a flask connected directly to an inverted measuring cylinder of oxygen provided with a leveling bulb containing water. The connection from flask to cylinder was all glass except for the rubber stopper. The latter was painted with

<div align="center">TABLE I</div>

<div align="center">*Rate of Oxidation of Cystine Solutions Exposed to Pure Oxygen*</div>

1.600 gm. of *l*-cystine in 320 cc. of solution at 38°; theoretical oxygen absorption for cysteic acid formation = 425 cc. (oxygen also at 38°).

Experiment No .	O₂ absorbed						
	1	2	3	4	5	6	7
	5 9 N HCl	5 9 N HCl + 10⁻⁵ mols Cu⁺⁺	5 9 N HCl + 10⁻⁴ mols Cu⁺⁺	5 9 N HCl + 10⁻³ mols Cu⁺⁺	5 9 N HCl + 10⁻⁴ mols Fe⁺⁺	5 0 N H₂SO₄ + 10⁻⁴ mols Cu⁺⁺	5.0 M H₃PO₄ + 10⁻⁴ mols Cu⁺⁺
days	*cc*	*cc*	*cc*	*cc*	*cc.*	*cc.*	*cc.*
11	20	30	30	50	10	0	0
56	30	35	65	170	20	10	0
92	45	55	100	250	30	15	5
120	55	60	115	295	35	20	5
168	60	70	145	365	45	20	5
200	65	75	160	395	45	20	5
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Cystine lost after 200 days as indicated by:							
O₂ absorption.....	15 3	17 6	37.6	93 0	10 6	4.7	1 2
Folin method. . .	1 0	8 5	27.5	81 3	0	5 0	0
Optical activity .	6 5	10 0	30 4	86 5	5 0	8 5	25 0

shellac. The oxygen in the cylinder was always kept at within a cm. of atmospheric pressure (in terms of water) in order to minimize the effect of small leaks. In practically all cases, however, there was a constant small loss of oxygen, emphasized particularly in the experiments at 38°, which ran over a much longer period of time (see loss percentages based on oxygen absorption).

The experiments in Table I were carried out entirely in a room

regulated at 38°, whereas those in Table II were done in an oven with the gas cylinder at room temperature.

The cystine used was a c.p. grade, several times recrystallized, which no doubt contained the usual small traces of heavy metals found in such cases unless especially purified reagents are used.

The figures for loss of cystine based on optical activity emphasize the fact that racemization proceeds in any event, even when

TABLE II

Rate of Oxidation of Cystine Solutions Exposed to Pure Oxygen

3.000 gm. of *l*-cystine in 600 cc. of 5.9 N HCl (1:1) at 60°; theoretical oxygen absorption for cysteic acid formation = 770 cc. (oxygen at room temperature).

Experiment No	O_2 absorbed				
	1	2	3	4	5
	No added catalyst	10^{-4} mols Cu^{++} added	10^{-2} mols Cu^{++} added	10^{-1} mols Cu^{++} added	10^{-4} mols Fe^{++} added
days	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
2	0	40	100	220	5
4	0	80	260	470	10
6	0	115	400	660	10
8	0	170	530	770	15
10	10	240	640	840	20
12	10	285	740	850	30
14	10	350	810	850	35
16	15	390	815		45
18		420	815		45
20		455	815		50
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Cystine lost after 20 days as indicated by:					
O_2 absorption..	2 0	59 0	100	100	6 5
Folin method..	0 5	54 0	100	100	2 0
Optical activity	15 0	66 5	100	100	58 0

little or no oxidation occurs. Only in cases of very rapid oxidation (Table II, Experiments 3 and 4) does the cystine oxidize nearly as rapidly as it racemizes. The cysteic acid isolated from these experiments has, moreover, always shown an optical activity lower than that obtained directly by bromine oxidation of *l*-cystine. Similar experiments with *dl*-cystine have produced inactive cysteic

acid at a rate identical with that of experiments in which *l*-cystine was used.   The configuration appears therefore to have no influence on the rate of oxidation and the two processes (racemization and oxidation) are quite independent.   The effectiveness of phosphoric acid in promoting racemization with little or no oxidation is again demonstrated by the loss in optical activity (Experiment 7, Table I).   The catalytic effect of copper in promoting this oxidation is evident from both Tables I and II.   It should be noted, however, that the halogen acid appears to be essential for its action; copper added to either sulfuric or phosphoric acids is entirely ineffective, and the use of these acids alone (with no copper added) gave results identical with those in Experiments 6 and 7, Table I.   The combination of copper and the halogen acid is evidently essential.

A series of experiments on other possible catalysts was run at 38°, but the values are omitted from Table I as the results were entirely negative.   The substances investigated included both ferrous and ferric salts, dried blood, hydroquinone, and similar substances. A series in which a combination of 10^{-4} mols each of copper and ferrous iron was added to the hydrochloric acid solution caused a somewhat lower speed of oxidation than when copper alone was used.

The excessive amounts of copper used in Experiments 3 and 4, Table II, demonstrate to what lengths the reaction can be pushed. In such cases, the very rapid oxygen absorption stops abruptly close to the theoretical point.   The mode of action of the copper probably consists in catalytic oxidation of small amounts of the halogen which in turn can easily oxidize the cystine.   Blank experiments in which the conditions in Experiment 4, Table II, were duplicated *without* addition of cystine showed no change in oxygen volume on standing several days.

Production of Inorganic Sulfate—It has previously been pointed out by the writer that inorganic sulfate is formed in varying quantities during the course of this oxidation (1), and examination of the solutions described in Tables I and II showed similar small amounts varying from almost none to about 10 per cent of the total sulfur present.   It was evident that in the more rapid oxidations there was much less sulfate formation than in the slower ones. The results thus lend support to the hypothesis that the sulfate is

formed by a side oxidation of some intermediate substance such as those described in a recent paper by Simonsen (2) and studied by Shinohara.[1]

This view is further supported by the complete failure in a number of experiments to obtain inorganic sulfate by oxidation of cysteic acid. It was thought that the splitting of sulfite, such as reported by Clarke and Inouye (3) in alkaline solutions of cysteic acid, might take place under these conditions also, leaving serine or pyruvic acid and making easily possible the further oxidation of sulfite to sulfate. However, solutions of both cysteic acid and its monosodium salt, both with and without copper, were recovered

TABLE III

Specific Rotation of Cysteic Acid

$C = 2.500$ gm. per 100 cc.; $T = 26°$.

Acid or base per mol cysteic acid	$[\alpha]_D^{25}$	Acid or base per mol cysteic acid	$[\alpha]_D^{25}$
mols	*degrees*	*mols*	*degrees*
33 HCl	+9.1	1 05 NaOH	−9 0
5 "	+8 5	1 1 "	−8 0
1 "	+8 0	1 2 "	−6 8
Isoelectric	+6 0	1 3 "	−5 8
0.25 NaOH	+3 2	2 0 "	−2 5
0.50 "	0	3 0 "	−0 6
0.75 "	−3 8	4 0 "	0
0.90 "	−6.5	6 0 "	+0.3
1.00 "	−8 4	7.5 "	+0 5

unchanged after standing 10 months at 38° in contact with oxygen. In this case, the use of the optical activity of the cysteic acid as one criterion of lack of decomposition led to some interesting results (see below) but the cysteic acid was unchanged and no inorganic sulfate was formed. More drastic attempts at oxidation, in which both the acid and its monosodium salt were exposed to an excess of free bromine for a week at 60° (with and without copper) also gave negative results. It appears evident that the sulfate is formed before the cysteic acid stage of oxidation is reached.

Optical Activity of Cysteic Acid—The use of the specific rotation

[1] Private communication from M. Kilpatrick and K. Shinohara of the Chemistry Department of this University.

of solutions of cysteic acid and of sodium monocysteinate as a criterion of decomposition necessitated a more complete investigation of the optical behavior of this substance when treated with varying amounts of acid or alkali.   The results are recorded in Table III.   For each determination, separate samples were weighed from the same lot of pure anhydrous cysteic acid.

The concentration used was uniformly 2.500 gm. of anhydrous acid per 100 cc. of solution.   Although similar optical activity curves have been reported for other amino acids, there appears to have been no previous record of such behavior on the part of cysteic acid.

SUMMARY

The oxidation of cystine in hydrochloric acid solution to cysteic acid by means of free oxygen has been further studied and found to be much accelerated by the presence of copper salts.

Copper is entirely ineffective in promoting this oxidation if sulfuric or phosphoric acid is used as solvent for the cystine.   In these acids no appreciable amount of oxidation appears to go on in any case.

All other substances tested for catalytic value in this reaction (ferrous and ferric salts, blood, and hydroquinone) were ineffective.

Phosphoric acid causes much more rapid racemization of cystine than do sulfuric and hydrochloric acids.

The curve of optical activity *versus* degree of neutralization of cysteic acid has been determined.

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THE DETERMINATION OF THYROXINE IN THE THYROID GLAND

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Leland and Foster (1) have recently reported a method for the determination of thyroxine in the thyroid gland which depends upon the selective extraction of thyroxine from the 2 N NaOH hydrolysate of the glandular material by means of butyl alcohol. Harington and Randall (2) have used butyl alcohol for the extraction of diiodotyrosine from the neutralized acid-soluble fraction of an alkaline hydrolysate of thyroid tissue. Several years ago we prepared butyl alcohol extracts of enzyme digests and of alkaline hydrolysates of thyroid glands for experimentation upon animals. Such extracts showed a marked physiological activity but also a high degree of toxicity. This was probably due to the pronounced solvent action of butyl alcohol for various peptides, amino acids, and tarry decomposition products. Other objections to the use of butyl alcohol for the preparation of organ extracts are its high boiling point and its tendency, under certain conditions, to form stubborn emulsions. It seemed for these reasons that ethyl acetate would be a more suitable extraction medium for thyroxine. Kendall (3) reported that the acid-precipitable fraction of an alkaline hydrolysate of thyroid glands is partly soluble in ethyl acetate. Experiments with this immiscible solvent showed that distribution ratios favorable for the differential extraction of thyroxine obtained only when the acidity of the liquid phase was adjusted to about pH 5. For various reasons, which are now being studied, the procedure did not prove entirely satisfactory.

When, however, such acid solutions containing thyroxine were shaken with butyl alcohol, it was found that in most cases fully 100 per cent of the suspended thyroxine passed into the organic

solvent in a single extraction. Although about 83 per cent of the diiodotyrosine and about 45 per cent of the inorganic iodine which was present in the solution passed into the butyl alcohol along with the thyroxine, the ease and completeness of the extraction of the latter at once offered a means for the simplification of the Leland and Foster technique. These authors reported distribution ratios between butyl alcohol and N or 2 N NaOH of 92:8.0 for thyroxine, 2.5:97.5 for diiodotyrosine, and 7.6:92.4 for potassium iodide. Because of these relationships, it was necessary to extract the alkaline gland solution with two successive portions of butyl alcohol, then wash the extract with alkali and lastly reextract the washings with butyl alcohol. The acid butyl alcohol extracts encountered in this work contain roughly 4 times as much iodine in the form of diiodotyrosine as of thyroxine. Theoretically, therefore, all that should be necessary in order to obtain essentially correct thyroxine iodine values in alkaline hydrolysates of thyroid tissue is to acidify the solution to a given pH, extract once with butyl alcohol, and wash the latter with an equal volume of 2 N NaOH. In practise, however, such a procedure gave quite irregular results. One reason for this may be the mechanical difficulty of removing the alkaline aqueous layer completely. Since this solution is now highly charged with iodine, its thorough separation becomes a matter of great importance. An alkaline solution for purifying the butyl alcohol extract was therefore devised which, because of its lesser solvent action for thyroxine, would permit of more than one washing of the butyl alcohol. With the introduction of this feature, the method proved capable of a high degree of accuracy. Among the advantages it offers are the absence of any necessity to transfer the gland solution from one separatory funnel to another, the whole process of extraction being completed in a single funnel, a greater economy of time and material, and the simultaneous extraction of most of the diiodotyrosine, which can be readily separated from the thyroxine fraction. Of some moment, perhaps, is the fact that the original mother liquid can now be quite easily freed from all of its iodine content and in this condition used for further work.

<div align="center">EXPERIMENTAL</div>

Distribution ratios for thyroxine, diiodotyrosine, and potassium iodide between butyl alcohol and an aqueous phase acidified with

sulfuric acid were determined in the presence of the products of hydrolysis of muscle proteins and the salt which would form upon the addition of the acid to 2 N NaOH. Dried and defatted beef muscle was boiled for 18 hours with 2 N NaOH in the proportion of 1.25 gm. of substance to 100 cc. of alkali, as recommended for thyroid by Leland and Foster. Weighed amounts of thyroxine, diiodotyrosine, and potassium iodide were dissolved in this in varying concentrations. Measured volumes of these solutions were transferred to separatory funnels, enough brom-cresol green indicator added to impart a distinct green color, and the solution titrated with 1:1 sulfuric acid to a distinct yellow color. The pH, as indicated by a test drop with brom-phenol blue, was near 3.5. While still warm, the turbid solution was shaken with a volume of butyl alcohol equal to that of the total volume of the aqueous layer and allowed to come to equilibrium at room temperature for from 60 to 90 minutes. After separation the residues of both layers were ashed for the determination of their respective iodine contents by the method of Kendall. In another series of experiments, aliquots of the butyl alcohol extracts which contained known amounts of the particular iodine compound under study were shaken with a solution of 4 N NaOH containing 5 per cent anhydrous Na_2CO_3. Distribution ratios between butyl alcohol and the alkaline mixture were thus found for the three iodine compounds of importance in this investigation. All the determinations were made at room temperature (22–28°).

On the basis of the data obtained (Table I) the following procedure for the determination of thyroxine in thyroid substance was worked out. Reflux 1.25 gm. of the dried, defatted gland for 18 hours with 100 cc. of 2 N NaOH in a Kjeldahl flask, as recommended by Leland and Foster. Cool the alkaline hydrolysate and transfer to a 300 cc. separatory funnel. Cool in running water to below room temperature, or chill in the ice chest. Add from 0.5 to 2.0 cc., or more, of brom-cresol green indicator (prepared according to Kolthoff (4)) so that the solution will assume a definite greenish tint. From a burette allow a 1:1 solution of H_2SO_4 to drop in with a moderate degree of rapidity until the color just changes to yellow, and then add about 0.2 cc. more. Note the volume of acid used. During the addition of the acid the solution will become quite warm and the funnel must be shaken vigorously and continuously in order to prevent the formation of a heavy floccu-

lent precipitate of the products of protein cleavage.   Remove a
test drop from the funnel with a fine-tipped glass rod and bring it
in contact with a small drop of brom-phenol blue indicator.   A pH
of 3.5 to 3.1 will usually be found.   Calculate the total volume of
the solution in the funnel.   To the Kjeldahl flask in which the
hydrolysis had taken place, add 1 or 2 drops of brom-cresol green
and drop into the bottom of the flask just enough acid to adjust
the reaction to about pH 3 (2 to 5 drops).   Measure into a gradu-
ate a volume of butyl alcohol equal to that (within 1 to 2 cc.) of
the total volume of the acid solution to be extracted.   Rinse the
Kjeldahl flask with small portions of the alcohol, and pour each

TABLE I

*Distribution Ratios of Thyroxine, Diiodotyrosine, and Potassium Iodide in
Presence of Sodium Sulfate and Products of Alkaline Hydrolysis of
Muscle Proteins*

Substance	Between butyl alcohol and H_2SO_4 at pH 3 8 to 3 1 Ratio of concentration in BuOH to concentration in H_2SO_4	Between butyl alcohol and 4 N NaOH containing 5 per cent Na_2CO_3 Ratio of concentration in BuOH to concentration in alkaline mixture
Thyroxine, average of 10 determinations	99 7:0 3	97 7:2 3
Diiodotyrosine, average of 7 determinations . . .	83 2:16 8	4 8:95 2
KI, average of 4 determinations	45.7:54 3	3 8:96 2

into the funnel until all has been added.   Shake vigorously while
still warm and set aside to cool to room temperature for from 60 to
90 minutes.   At the end of that time draw off the light straw-
colored aqueous layer as thoroughly as possible, leaving any slight
amount of tar or interfacial precipitate which may be formed in
the butyl alcohol.   Add now a volume of 4 N NaOH containing
5 per cent anhydrous sodium carbonate equal to that of the volume
of butyl alcohol taken, and shake for a few minutes.   Allow to
stand for an hour, or 90 minutes.   Draw off the dark brown alka-
line solution to the last drop   Shake the butyl alcohol remaining
in the funnel with a volume of the sodium hydroxide-carbonate
mixture equal to one-half the volume of butyl alcohol originally

taken. After an hour or longer, separate well, and wash the end of the funnel below the stop-cock with distilled water. Run the butyl alcohol into a distilling flask of suitable size. Rinse the funnel with several small portions of butyl alcohol and add to the distilling flask. Add a few drops of concentrated NaOH and distil off the butyl alcohol from a water bath at a low pressure. Allow the distillation to continue nearly to dryness. Wash the residue with distilled water and the aid of dilute NaOH into a 250 cc. nickel crucible. Evaporate over a sand bath or hot plate to dryness. Ash and analyze for iodine as in the method of Kendall.

<div align="center">DISCUSSION</div>

The work of Leland and Foster (1) has shown that the alkaline hydrolysis of thyroids as used in their method may cause a destruction of thyroxine to the extent of about 17 per cent. We are now conducting experiments with a view of finding a more satisfactory method for freeing the thyroxine from its combination with thyroid protein. The analytical procedure detailed above gave consistent results in a large series of determinations of thyroxine in thyroid substance from various sources. Duplicate determinations as a rule agreed within 2 per cent. The method is not critical as to the degree of acidity, below a pH of about 3. Twice the amount of acid required to reach this level of the hydrogen ion concentration may be added without any deleterious influence upon the final thyroxine iodine value. It is best, however, to avoid an undue excess of acid, as the portion that passes into the butyl alcohol, (about 8 parts of acid per 100 parts of BuOH) and that inevitably remaining in the separatory funnel after the aqueous layer is drawn off, may neutralize to an undesirable extent the alkali which is used to wash the butyl alcohol extract. The only crucial step in the technique seems to be the manner of adding the acid to the strongly alkaline gland solution. If the solution in the funnel is not well cooled and is not thoroughly shaken, especially if the acid is being added too rapidly, a heavy gelatinous precipitate will be formed which will hopelessly emulsify with the butyl alcohol and from which it will be found rather difficult to extract the thyroxine quantitatively. On the other hand, it is to be noted that a precipitate begins to form at about pH 5, which largely redissolves on the continued addition of tl e acid, provided

that the acid is added fast enough to pass this precipitation range rather rapidly.

The method has been tested under a variety of conditions. In each instance quantitative recoveries of thyroxine iodine were obtained. The ratio of diiodotyrosine and of potassium iodide to thyroxine seems to have no significant influence upon the final thyroxine figures. Correct values for thyroxine iodine were also found when the latter was added to thyroid solutions either before or after the extraction therefrom of the native thyroxine by the proposed method. The results are shown in Table II.

Comparison with Method of Leland and Foster

The thyroid glands used in these experiments were, for the most part, goitrous glands removed from human subjects. A multiple of 1.25 gm. (2 to 5 × 1.25) of the dried and defatted substance was hydrolyzed in an appropriate volume of 2 N NaOH. The solution was made up to volume and aliquots were analyzed for their thyroxine content by the two methods. The results in Table III show that the extraction with butyl alcohol of an acid solution of thyroid yields consistently higher values for thyroxine iodine than the Leland and Foster method. In view of the fact, however, that these authors reported large losses of thyroxine (about 17 per cent) occasioned by the method of hydrolysis employed, these differences are of no great consequence as far as the actual thyroxine content of the gland is concerned, since in terms of percentage they very rarely exceed the extent to which destruction of thyroxine is supposed to take place. It does not seem, however, that the higher values by the new method are due to any intrinsic defect in the procedure. This view is prompted both by theoretical considerations based upon the distribution ratios in Table I and the actual results of experiments bearing upon this point. Table IV shows the extent to which each iodine compound may contribute its quota to the final thyroxine iodine value obtained in the butyl alcohol extracts. The figures are in good agreement with the theory and lie well within the limits of error of the iodine method. They suggest strongly that our thyroxine values are not the result of an adventitious mixture of the three forms of iodine occurring in the gland solution, but rather that these are made up of the specific proportions indicated in Table IV. The

error seems to fall largely upon the diiodotyrosine, but, as a calculation will show, only in the extreme case of a ratio of diiodotyrosine to thyroxine of 10:1 can this error even approximate the magnitude of +5 per cent. That the amounts of iodine found in the butyl alcohol extracts are not the outcome of a fortuitous balancing of errors is further indicated by the fact that duplicate deter-

TABLE II

Recovery of Thyroxine from Butyl Alcohol Extracts of Mixtures of Thyroxine, Diiodotyrosine, and KI Dissolved in 2 N NaOH Hydrolysate of Muscle Proteins

Composition of solution						Thyroxine I found	
Inorganic I		Diiodotyrosine I		Thyroxine I			
Total	Per cent of total I	Total	Per cent of total I	Total	Per cent of total I	Total	Per cent of thyroxine I taken
mg.		*mg*		*mg*		*mg*	
0 086	2 4	3 12	87 1	0 377	10 5	0 375	99 5
0 408	7 70	4 03	76 3	0 853	16 0	0 870	101 6
0 433	7.97	4 25	78 0	0 763	14 03	0 763	100 0
0 433	7 99	4 25	78 0	0 763	14 03	0 751	98.5
0 433	7 99	4 25	78 0	0 763	14 03	0 765	100 0
0 0	0 0	4 43	85 30	0 761	14 70	0.759	99 5
0 0	0 0	4 43	85 30	0 761	14 70	0 761	100 0
0 0	0 0	4 43	85 30	0 761	14 70	0 755	99 3
0 0	0.0	4 43	85 30	0 761	14 70	0 761	100 0
0 0	0 0	4 43	85 30	0 761	14 70	0 757	99 5
0 0	0 0	11 64	94 70	0 649	5 30	0 668	103 0
0.0	0 0	5 87	90 03	0 649	9.97	0 658	101.3
0 0	0 0	1 17	64 40	0 649	35 60	0 648	100 0
0 0	0.0	0 353	52 00	0 325	48 00	0.320	98 6
0 0	0.0	4 69	93 60	0 325	6.40	0 327	100 5
2.865	43 30	2 710	41 02	1.037	15.68	1 045	101 0
2.865	43 30	2 710	40 02	1 037	15 68	1 033	99 7

minations of thyroxine by the procedure here described always agree within the limits of error of the iodine estimations.

Some comment seems to be called for with respect to the possible effect of inorganic iodine upon the thyroxine estimations, since from an acid solution butyl alcohol removes more than 40 per cent of the iodine present in this form. Leland and Foster found about 8 parts of KI in the butyl alcohol layer after the latter

was shaken with a 2 N NaOH solution of the substance.   With
the stronger alkali used in the new method the distribution ratio

TABLE III

*Comparison of Results with Those Obtained with Method of Leland and Foster
in Thyroid Substance*

| | | Thyroxine iodine | | | | |
| | Total I₂ per gm. dry gland | | | As per cent of total I₂ | | Differ-ence |
Material		Leland and Foster method	Proposed method	Leland and Foster method	Proposed method	
	mg.	*mg.*	*mg.*			*per cent*
Human thyroid.	3.31	0.56	0 618	16 90	18.70	+10.(
" "	1.23	0 367	0.391	29.90	31.80	+6.(
" "	1.278	0 0735	0.0788	5.75	6.16	+7.(
" "	1 21	0 076	0.085	6 30	7.00	+11.(
" "	4 04	0 778	0.887	19.30	22 00	+14.(
" "	0.720	0.0127	0 0127	1.77	1.77	0 (
Pig thyroglobulin........	7.28	1 880	2.230	25 80	30.60	+18 (
" "	6.00	1.44	1.680	24.00	28.00	+17.(
Desiccated pig thyroid.....	4.08	0.96	1.120	23.50	27.40	+16 !

TABLE IV

*Iodine Compounds Remaining in Butyl Alcohol after Treatment of Extract
with Alkali*

Substance	I₂ taken	I₂ found in BuOH	
	mg.	*mg.*	*per cent*
Thyroxine	0.957	0.933	97.4
	0.957	0.927	97.0
	0.957	0.939	98.0
Diiodotyrosine	2.36	0 015	0.63
	2.775	0.013	0.60
	2 085	0.0114	0.55
KI	4.56	0.006	0.13
	4.56	0 009	0.19

favors more decidedly the aqueous layer, and, as shown in Table
IV, leads to a more thorough removal of the inorganic iodine from
the butyl alcohol.   In this connection it may be mentioned that
the statement of Leland and Foster that the concentrations of

inorganic iodine in thyroid hydrolysates can be but small, needs qualification, inasmuch as their own work seems to show that the amount of inorganic iodine originally present in the gland is augmented somewhat by the decomposition of thyroxine and, according to Harington and Randall (2), is increased to a much greater extent by the iodine split off from the diiodotyrosine.

In the comparison of the thyroxine values by the two methods (Table III) it is interesting to note that the divergence between the figures is smallest in pathological glands of low total iodine, and of relatively still lower thyroxine content; and greater by far in thyroglobulin and desiccated thyroid substance, where, presumably, normal relationships prevail between total iodine and iodine present in the form of thyroxine.   The significance of this is not now clear.

The discrepancy in the results by the two procedures as shown in Table III may be explained in at least two ways.   One would be the occurrence in the thyroid gland of a hitherto unidentified iodine compound which is more soluble in acid butyl alcohol than in alkaline butyl alcohol.   Another possibility is that thyroxine itself may be present in the alkaline hydrolysate in more than one form, some of which may be insoluble or soluble only to a limited extent in butyl alcohol.   When, however, the thyroxine is precipitated with sulfuric acid, the entire quantity, irrespective of its original form, is converted into the sulfate of thyroxine and thus presents itself as a single and uniform chemical entity for extraction by the butyl alcohol.

We are continuing the study of the preparation of iodine fractions of the thyroid gland by extraction with various organic solvents for chemical and biological investigations.

SUMMARY

A method is presented for the quantitative extraction of thyroxine from alkaline hydrolysates of thyroid substance, which depends upon the insolubility of thyroxine in an acid solution and upon the pronounced solubility of the acid salt of the product in butyl alcohol.

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THE ANALYSIS OF CHLORIDE IN TISSUES

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In a former paper (1) it was shown that certain biological materials cannot be analyzed for chloride with satisfactory results by the use of the direct open Carius method as applied by either Van Slyke (2) or by Wilson and Ball (3) for the analysis of chloride in blood or serum. In the procedure which is here reported a preliminary alkaline digestion is employed before the addition of $AgNO_3$ and HNO_3 and has been found to be a convenient method for the measurement of chloride in either dried or wet tissues.

Procedure

1 to 2 gm. quantities of finely hashed and thoroughly mixed wet tissue or 1 gm. quantities of pulverized dried tissue are placed in 125 ml. Erlenmeyer flasks and covered with 15 ml. of N KOH. The contents of the flasks are digested over a steam bath for $1\frac{1}{2}$ hours, being swirled occasionally in order to saponify any fats floating on the surface. Blank analyses are set up for each series and treated in the same manner as the tissues. After cooling to room temperature, 2 drops of methyl orange indicator are added to the contents of each flask. Starting with the blank analyses, add approximately 6 N HNO_3 drop by drop with gentle agitation to the turning point of the indicator and note the amount of acid required to produce the color change.

The color of methyl orange is frequently masked in flasks containing tissue, in which case the same quantity of HNO_3 is added to the contents of these flasks as is required for the blanks. A slight excess of aqueous $AgNO_3$ solution,[1] depending upon the approximate quantity of chloride present in the tissue, and 5 ml. of con-

[1] It is convenient to have prepared 0.15, 0.20, and 0.25 N $AgNO_3$ solutions.

centrated HNO_3 are added to each flask.   A rough preliminary analysis is often necessary to determine the amount of $AgNO_3$ to be used, since restriction of the amount to a slight excess secures a sharper end-point in the final titration.   The contents of the flasks are digested over a steam bath for 2 to 3 hours until the AgCl is well coagulated, the fatty acids are floated on the surface, and the intervening solution is clear.   The solutions are then filtered and the contents on the filter paper are washed carefully and thoroughly with hot water.   The solutions are cooled to room temperature and a saturated solution of ferric alum is added in the proportion of 2 ml. per 100 ml. of solution.   The volume of solution usually present for analysis is approximately 40 ml.   The blank and tissue solutions are then titrated with previously standardized 0.02 N NH_4CNS[2] until the first definite faint pink coloration persists after shaking.

The chloride of the tissue is calculated from the difference between the titrations of the blank and of the specimen.

Tests of the Method

Comparison with the Direct Open Carius Method—In Table I are given the results of our measurements of different tissues and feces in which the chloride was analyzed by the open Carius method and by the preliminary alkaline digestion procedure as specified in this paper.   The results represent the average of triplicate measurements.   Drying of the tissues was accomplished by heating in an oven at 100–110° for 48 hours.   It will be seen that higher measurements of chloride were consistently obtained with the preliminary alkaline digestion procedure than with the direct open Carius, and that after alkaline digestion the measurements were essentially the same for both the wet and the dried samples. Greater recovery of chloride was also obtained with the alkaline digestion of dried feces than by the direct open Carius procedure.

Addition of NaCl to Tissues—Known amounts of NaCl were added to weighed amounts of dried and wet tissues and analyses were made by the direct open Carius and alkaline digestion pro-

[2] The NH_4CNS solution changes slowly on standing, so that frequent standardizations are necessary.   We standardized the solution for each day's analysis by control titrations of weighed amounts of proof silver dissolved in concentrated HNO_3, with 5 per cent ferric alum as the indicator.

TABLE I

Chlorides in Tissues and Feces

The results are expressed in milli-equivalents of Cl per kilo of wet sample, in each case.

Material	Direct open Carius method*		Alkaline digestion method	
	Wet samples	Dried samples	Wet samples	Dried samples
Beef muscle	13 6	12 1	14 3	14 5
" liver	32 0	28 9	34 2	34 5
" Kidney 1.	69 7	61 0	74 6	74 3
" " 2	54 1	43 4	60 2	59 3
Chicken skin.	23.2	19 0	26 8	26 6
Calf brain	40 5	33 7	44 4	45 0
Beef heart	22 4	19 0	23 7	24 9
" lung	55 0	46 5	58 1	58 6
Feces		1 87		2 97

* AgNO₃ and HNO₃ were added separately. Titrations with NH₄CNS were made in the presence of AgCl.

TABLE II

Chloride Content of Tissues Measured with and without Added NaCl

The results are expressed in milli-equivalents of Cl per kilo of wet sample, in each case

Material	Direct open Carius method Wet samples	Alkaline digestion method	
		Wet samples	Dried samples
Weight of samples, 0.8-1.5 gm.			
Kidney 2	54 1	60 2	59 3
" + 1 ml. NaCl 0.0195 M	56 7	59 7	60 6
" + 1 " " 0.0739 "	59 1	60 6	60 1
Weight, 1.4-3.0 gm			
Beef heart	22 4	23 7	24 9
" " + 1 ml. NaCl 0.0251 M	23 2	24 2	25 4
" " + 1 " " 0 0495 "	25 6	24 5	25 4
Weight, 0.9 1 9 gm.			
Beef lung	55 0	58 1	58 6
" " + 1 ml. NaCl 0.0241 M	57 1	60 6	58 9
" " + 1 " " 0.0738 "	55 6	59 1	58 7

* The results represent the average of triplicate analyses. The values for each analysis have been obtained by deducting the chloride added to the tissues from the chloride measured analytically.

cedures. The results of the analyses of wet tissues by the open Carius method and of wet and dried tissues with which preliminary alkaline digestion was employed are given in Table II and have been expressed in terms of milli-equivalents of Cl originally present per kilo of wet tissue. Thus, in the analyses with added salt, the added chloride is deducted from the observed value to obtain the value given in Table II. It will be seen that the chloride of the original tissue measured by the direct open Carius method when analyzed with or without the addition of chloride shows greater variation than when measured after preliminary alkaline digestion.

TABLE III

*Chloride Analysis of Tissues**

The results are expressed in milli-equivalents of Cl per kilo of dried material.

Material	Direct open Carius method	Alkaline digestion method	Thompson-Oakdale method	Lemp-Broderson method
Bacto-Liver	88 8	98 3	98 9	
Bacto-Veal	106 9	124 8	122 5	122 3
Chicken skin . .	49 6	72 0	73 3	
Calf brain .	170 9	193 5	191 1	191 1
Beef lung . .	263 4	274 4	270 5	276 9
" Kidney 3	317 6	336 0	334 6	336 9

* The gravimetric analyses were made in duplicate; the titrimetric analyses were made in triplicate.

Comparison with Other Methods of Decomposition—Chloride analyses of tissues by means of titration after the direct open Carius and after preliminary alkaline digestion procedures were compared with analyses made by means of modifications of the Lemp-Broderson (4) and Thompson-Oakdale (5) gravimetric methods. Commercial Bacto-Liver, Bacto-Veal, and dried, pulverized tissues which had been sifted through 30-mesh copper screening were used for these analyses (Table III).

The destruction of the organic material in the Lemp-Broderson method was effected in an electrically ignited Burgess-Parr bomb. 1 gm. quantities of the material for analysis were thoroughly mixed by means of a glass stirring rod with 1 gm. of finely ground potassium nitrate and 15 gm. of sodium peroxide. With certain

tissues containing a large percentage of fat, the tissue particles tended to coalesce. When equal parts of such tissues and potassium nitrate were ground together, there was less cohesion and the resulting mixture was readily miscible with the sodium peroxide. In all cases special care was taken to exclude water in order to guard against spontaneous ignition of the organic material with the sodium peroxide. This charge yielded a satisfactory fusion which was practically free of amorphous carbon. The melt was dissolved in water and from three to six melts were pooled together for each analysis. After neutralizing with concentrated HNO_3 and filtering to remove the flaky carbon, $AgNO_3$ in excess and concentrated HNO_3 were added. The solutions were protected from light and permitted to stand overnight at a temperature of 50°. After cooling, the supernatant liquid was decanted from the firm white precipitate of AgCl which collected at the bottom. The solutions containing the precipitate were then filtered through crucibles with porous porcelain bottoms which had been cut down to a height of 15 mm. and which weighed from 5 to 6 gm. After washing the precipitate with 1 per cent HNO_3 and water, the crucibles were heated to constant weight in a muffle furnace at 280°. Commercial reagents of the highest purity were used throughout. The blanks, which were independently determined and deducted, contained approximately 1.5 mg. of AgCl per charge. The amount of AgCl weighed for each analysis was between 100 and 200 mg.

For the Thompson-Oakdale method chromic acid with fuming H_2SO_4 was used as the oxidizing agent in the digestion of tissues. To the Kjeldahl flask of the Thompson-Oakdale apparatus was joined a side arm attachment to facilitate the introduction of tissue and reagents. The liberated chlorine was absorbed and reduced to chloride in an alkaline solution of sodium arsenite (prepared by dissolving 1 gm. of arsenic trioxide in 100 cc. of 10 per cent chlorine-free NaOH) and determined gravimetrically in the same manner as with the Lemp-Broderson method.

In Table III are given the results of the analyses of tissues made by the gravimetric and titrimetric methods. It will be seen that the results obtained with the alkaline digestion procedure agreed within less than ±2 per cent with those obtained by either the Thompson-Oakdale or Lemp-Broderson method. The results of

the analyses made by the direct open Carius method upon dry materials were from 4 to 32 per cent less than those made with the other procedures.

Remarks—The question arises as to whether maximal recovery of chloride is obtained in lipemic blood with the direct open Carius method.   The sera from two patients suffering with lipid nephrosis were analyzed for chloride by the Wilson and Ball procedure with and without previous alkaline digestion.   The results of these analyses by either procedure were practically identical for the respective sera.   We have no evidence to suggest that the open Carius method according to the Wilson and Ball procedure applied to wet blood does not yield maximal recovery of chloride.

Andrews (6) believed that the presence of sulfate interfered with the precision of the Volhard titration in certain instances because "appreciable amounts of difficultly soluble silver sulphate are dragged down with the chloride and sulphocyanate." Since tissues contain relatively larger amounts of sulfate than blood, chloride analyses were made on serum to which Na_2SO_4 (in 1 ml. amounts of 50 and 100 mM Na_2SO_4 solutions per 1 ml. of serum) was added. The measurements of chloride were practically identical in the serum with or without the added sulfate.

In order to secure the most reliable end-point with the Volhard titration of tissue solutions, it is our experience that the titrations should be made in filtered solutions which contain just sufficient $AgNO_3$ in excess so that no more than 2 ml. of 0.02 N NH_4CNS are required for the final titration.   The formation of much $AgCNS$ in the yellow-colored solutions of tissues is apt to mask the delicate end-point.   No empirical correction for the reading of the end-point was required for our analyses.   Titrations of unfiltered solutions of tissues, even though the AgCl was well coagulated, generally gave fading or indistinct end-points,—a difficulty not encountered in the analysis of blood or serum.   Various reagents such as acetone, ether, and alcoholic thiocyanate solution have been advocated for sharpening the end-point.   No particular improvement was noted with the use of these reagents.

SUMMARY

Complete recovery of chloride in tissues and feces was not obtained by the use of the direct open Carius method.   A pro-

cedure for analysis with preliminary alkaline digestion followed by the open Carius method has been developed which gives results agreeing within ±2 per cent with those obtained by established methods for analysis of chloride in organic materials.

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THE ISOLATION AND CHARACTERIZATION OF MESOCYSTINE*

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In a previous communication, the isolation of two optically inactive isomers of cystine from the inactive product produced by the racemization of *l*-cystine with boiling hydrochloric acid was announced (1). One of the isomers was shown to be the racemic modification and the other was tentatively assigned the meso or internally compensated structure. It is the purpose of this communication to present the method of isolation and proof of structure of the mesocystine and further certain additional data on *dl*-cystine, the isolation of which has already been presented in detail (2).

The actual isolation of both *dl*- and mesocystine in pure crystalline form from inactive cystine brings to a close the long controversy as to whether this inactive cystine, first described by Mörner (3) in 1899, represented the meso or racemic form or whether both isomers might be present.

The isolation of these isomers in pure form, furthermore, makes possible the use of definitely characterized isomers in physiological studies of the inactive cystines. The ease of isolation of the racemic isomer should eliminate the use of the poorly characterized mixture of isomers that has hitherto been used in studies purportedly on *dl*-cystine.

Fractionation of the Inactive Cystine Hydrochloride—In a previous publication (2) the hydrochloride of *dl*-cystine was isolated directly from the hydrochloric acid solution after heating the *l*-cystine with 20 per cent HCl by concentrating the solution *in vacuo*. Fractions of the *dl*-cystine hydrochloride were removed and purified and, as stated, the mesocystine hydrochloride could be recovered from the mother liquors.   We have since found that the mesocystine can be more readily isolated and larger yields obtained if, instead of working with the original acid solution, the free cystines are first isolated and then dissolved in HCl and fractionated.

100 gm. of *l*-cystine were dissolved in 1 liter of 20 per cent HCl and refluxed for 5 days.   After the solution was partially decolorized by boiling with approximately 10 gm. of carboraffin, it was concentrated *in vacuo* until considerable solid material had separated.   The mixture was then dissolved in approximately a liter of water and made neutral to Congo red paper by the slow addition of concentrated NH₄OH.   The precipitated cystine was then dissolved in a minimum of 10 per cent HCl, diluted to 700 cc. with water, and decolorized with carboraffin.   The almost colorless solution was again neutralized as before, and allowed to stand in the ice box for 24 hours to complete the precipitation.   The yield of inactive cystine usually represented about 60 per cent of the amount of *l*-cystine started with.   The content of unchanged *l*-cystine in this product was estimated to be in the neighborhood of 1 per cent.

The above inactive cystine was then dissolved in approximately 200 cc. of 20 per cent HCl and concentrated *in vacuo* at 40–50° until crystals began to form.   The solution was then cooled to room temperature and, after standing for an hour or so to allow time for crystallization, it was filtered.   The crystals, which amounted to about 30 gm., were washed with an ice-cold mixture of equal parts of alcohol and ether containing 5 cc. of concentrated HCl per 100 cc. of solution.   The washings were added to the mother liquor which was again concentrated to the crystallization point and treated as above.   This second fraction usually amounted to about 10 gm.

Two recrystallizations of Fraction I and three or four for Frac-

tion II usually sufficed to give pure *dl*-cystine hydrochloride.   The
recrystallizations were carried out by dissolving the hydrochloride
in 6 times the amount of water with enough concentrated HCl
to keep the cystine in solution and then adding to 2 parts of this

Fig. 1
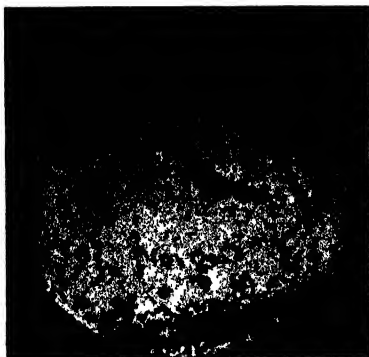
Fig 2

Fig. 3

Fig. 4

Fig. 1.   *dl*-Cystine hydrochloride (50 ×).
Fig. 2.   Mesocystine hydrochloride (50 ×).
Fig. 3.   *dl*-Cystine (110 ×).
Fig. 4.   Mesocystine (100 ×).

solution, 1 part of concentrated HCl.   After the solution had stood
for a short time, glistening diamond-like platelets of *dl*-cystine
hydrochloride separated (Fig. 1).   The yield of the final purified
product from both fractions varied from 15 to 25 gm.

The filtrate and washings from Fraction II were further concentrated to a syrup and after standing the syrup crystallized, yielding short blunt prismatic crystals of the type shown in Fig. 2. These crystals were filtered off and represented Fraction III.   This
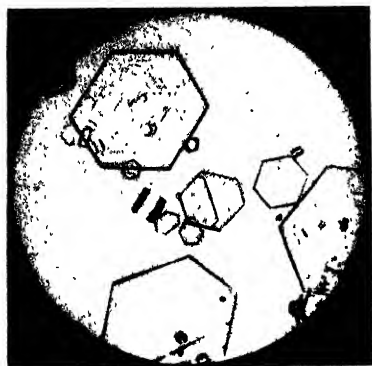
FIG. 5
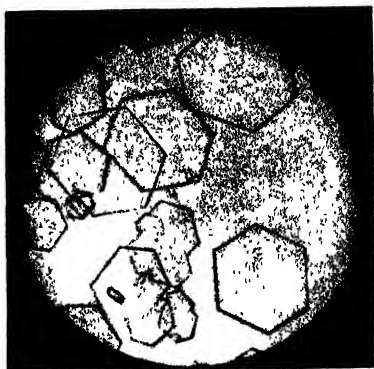
FIG. 6

FIG. 7

FIG. 8

FIG. 5.   *d*-Cystine (50 ×).
FIG. 6.   *l*-Cystine (50 ×).
FIG. 7.   *d*-Cystine hydrochloride (50 ×).
FIG. 8   *l*-Cystine hydrochloride (50 ×).

fraction usually amounted to 20 to 25 gm.   With larger runs a fourth fraction consisting of these prismatic crystals could usually be obtained from the mother liquor of Fraction III.

It was found that the crude mesocystine hydrochloride (Fraction III) could not be recrystallized advantageously by the same procedure used for the racemic fraction.   The procedure found to be most satisfactory was to dissolve the crude mesocystine in 10 per cent HCl, concentrate the solution *in vacuo* to a syrup, and then bring about crystallization of the mesocystine hydrochloride by vigorously rubbing with a glass rod the sides of the beaker to which the syrup had been transferred.   Crystallization takes place very slowly.   When the mixture had reached a mush-like consistency, the crystals were filtered and washed as in the case of the racemic fractions.

The number of recrystallizations varied somewhat with the individual run.   Three recrystallizations were found, usually, to yield a mesocystine hydrochloride of sufficient purity so that on conversion to free cystine, which affords further purification, pure mesocystine can be obtained.   It is well to follow the crystallization for uniformity of crystalline structure by microscopic examination.   This is of great assistance in following the purification, although this is not in itself a sufficient criterion of the degree of purity.   We have found that fractions which were apparently pure by microscopic examination were actually not pure as indicated by solubility determinations of the free cystine prepared from them.   The analytical data on nitrogen and sulfur indicated that no impurity other than isomeric cystines was likely to be present. The lack of purity was shown by the solubility which varied, depending on the proportion of solid phase present.   The method of determining the solubility and the solubility data will be presented later in a detailed solubility study of all four isomers of cystine.   Our criterion of purity was therefore constant solubility, regardless of the proportion of solid phase used, in addition to uniformity of crystalline form and analytical data.   This criterion was also used for the racemic isomer as well.   The yield of the purified mesocystine hydrochloride was small as there is a considerable loss in the recrystallizations.   After three recrystallizations of Fraction III about 8 to 10 gm. are ordinarily obtained.

Attempts to obtain further crops of mesocystine by reworking the mother liquors from the above recrystallizations were only moderately successful.   Although at times the yields of mesocystine could be thus increased, in many cases, instead of obtaining

characteristic crystals of either *dl*-cystine or mesocystine hydrochloride, we obtained material crystallizing in whetstone-shaped crystals or in very tiny needles that were refractory to further fractionation. From some preliminary work we have been led to suspect that such crystals might represent mixed crystals of the cystine isomers. This is, however, merely a supposition and we hope to investigate further such preparations. It might be added that the cystine prepared from such a preparation always showed a higher solubility than normal.

Isolation of the Free Meso- and dl-Cystines—To obtain the free cystines in well defined crystalline form, it was found best to utilize the method which Emil Fischer used for crystallizing *l*-cystine; that is, dissolving the hydrochloride in sufficient water and allowing the cystine to separate spontaneously. The optimum amount of water, however, differed considerably for the two inactive isomers.

In the case of the racemic isomer 10 gm. of our purified hydrochloride were added slowly to 2500 cc. of water and dissolved by shaking the mixture. After a few minutes elongated hexagonal prisms (Fig. 3) began to precipitate and after 24 hours approximately 87 per cent of the cystine had separated. This product was filtered and washed until free from chlorides. A second crop of *dl*-cystine can be obtained by concentrating the mother liquor *in vacuo* and neutralizing the solution with NH_4OH. The analytical data for *dl*-cystine have already been given (2).

Pure mesocystine was prepared similarly except that considerably less water was used. 10 gm. of the hydrochloride were dissolved in 850 cc. of water and the solution allowed to stand for 24 hours. The mesocystine precipitated in long narrow parallelogram-like plates as indicated in Fig. 4. The material was filtered and washed with water until free from chlorides. About 55 per cent of the cystine was so obtained. Another crop could be obtained, although less pure, by concentrating the mother liquor and neutralizing the solution with NH_4OH.

The mesocystine decomposed over a range of 200–218° and a 1 per cent solution in 1 N HCl failed to show any rotation. Analyses for nitrogen and sulfur agreed with the expected values for an isomeric cystine.

Analysis

3.232 mg. substance: 0.348 cc. N at 33° and 750 mm.
5.111 " " : 9.91 mg. BaSO₄

<div style="margin-left:2em">

$C_6H_{12}O_4N_2S_2$. Calculated. N 11.67, S 26.66
Found. " 11.82, " 26.62

</div>

Diformylmesocystine —The diformylmesocystine was prepared by the method already described for diformyl-*dl*-cystine (2). The meso derivative melted at 192–193° (corrected) and crystallized in parallelogram-like plates.

Analysis

2.948 mg. substance: 0.253 cc. N at 31° and 757 mm

<div style="margin-left:2em">

$C_8H_{12}O_6N_2S_2$. Calculated, N 9.46; found, N 9.57

</div>

The racemic compound melted slightly higher, 196–198°, and crystallized in rectangular prisms often with the ends cut to form elongated hexagonal crystals A mixture of the two melted at 189–190°.

Proof of the Stereo Structure of Mesocystine In order to demonstrate that the isomeric cystine which was believed to be the meso isomer was truly the internally compensated form, it was necessary to show that it could not be resolved by procedures that lead to the resolution of the racemic compound. The brucine salt of the diformylmesocystine was prepared and upon recrystallization no change in rotation occurred. Under the same conditions recrystallization of the brucine salt of the *dl*-formylcystine led to resolution. The salt obtained from the meso derivative was extremely soluble, however, and it was necessary to work with very syrupy solutions in order to crystallize it. The results were therefore not entirely satisfactory. Other alkaloidal salts were therefore investigated and it was found that the strychnine salt crystallized beautifully. As previously described (2), the *dl*-formylcystine could be readily resolved by means of the strychnine salt to yield *d*- and *l*-cystine (Figs. 5 and 6), the hydrochlorides of which are shown in Figs. 7 and 8. Under the same conditions no resolution of the strychnine salt of the suspected meso compound could be obtained, affording proof that it was the internally compensated form.

2.6 gm. of diformylcystine and 6 gm. of powdered strychnine

were added to 30 cc. of boiling water and the solution filtered from
any undissolved strychnine. After cooling, a good yield of the
salt was obtained. The product was crystallized three times, the
yield of the salt from the last crystallization being 2.5 gm. The
rotation was determined at each stage. The original rotation was
$[\alpha]_D^{26.5} = -19.5°$ and the salts from the successive recrystalliza-
tions gave respectively the following rotations: $[\alpha]_D^{27} = -20.0°$,
$[\alpha]_D^{26} = -20.5°$, and $[\alpha]_D^{27.5} = -20.5°$. As a further check on the
uniformity of the product all the mother liquors were combined,
evaporated to dryness, and the residue crystallized from the mini-
mum amount of water. A value of $[\alpha]_D^{26} = -21.5°$ for this sample
was obtained.

The strychnine salt of diformylmesocystine crystallized with 4
molecules of water of crystallization and decomposed at 133–135°
(corrected). The compound, after being dried *in vacuo* over P_2O_5,
decomposed at 175–176°.

Analysis
2.990 mg. substance: 0.240 cc. N at 28° and 746 mm.
$C_{50}H_{56}O_{12}N_6S_2$. Calculated, N 8.72; found, N 8.93

The diformylmesocystine obtained from the purified strychnine
salt possessed the same decomposition point and crystalline struc-
ture as the original compound.

Phenyl Isocyanate Derivatives—1 gm. of the mesocystine hydro-
chloride was dissolved in 16 cc. of water and 17.33 cc. of 1 N NaOH
were added to the solution cooled in an ice bath. 1 cc. of phenyl
isocyanate was then added and the mixture shaken in the ice bath
until the odor of the isocyanate had disappeared. The solution
was diluted with 5 cc. of water and filtered. The phenyluramino
derivative was precipitated by acidifying the filtrate with HCl. It
was then recrystallized from 95 per cent alcohol. The compound
melted at 191–192° (corrected).

Analysis
3.332 mg. substance: 0.341 cc. N at 26.5° and 744 mm.
2.961 "   "   : 0.312 " " " 27.5° " 740 "
$C_{20}H_{22}O_6N_4S_2$. Calculated, N 11.7; found, N 11.42, 11.66

The phenyluramino derivative of *dl*-cystine was prepared in
similar fashion. It melted at 195–196° (corrected).

Analysis

2.814 mg. substance: 0.299 cc. N at 27° and 744 mm.

3.188 "   "   : 0.341 " " " 28° " 738 "

$C_{20}H_{22}O_6N_4S_2$. Calculated, N 11.7; found, N 11.83, 11.78

SUMMARY

The isolation of the internally compensated stereoisomer of cystine, mesocystine, has been described with proof of the stereo structure of the compound offered.

Further data on the isolation of *dl*-cystine have also been presented.

Various derivatives of the two optically inactive isomers of cystine have been prepared.

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OPTICAL ROTATIONS OF CONFIGURATIONALLY RELATED METHOXYPROPIONIC AND β-METHOXYBUTYRIC NITRILES

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The investigations into the rotatory dispersions of configurationally related substances containing 1 asymmetric carbon atom of the general type of $H - C\begin{smallmatrix} R_1 \\ | \\ | \\ R_2 \end{smallmatrix} - (CH_2)_n - X$ (in which $n = 0$ or any integer), which have been carried out in this laboratory, have brought out the significance of the value n on the direction of the rotatory contribution of the group X. In other words, it was found that an identical group X may furnish a levorotatory or a dextrorotatory contribution depending on its being attached directly to the asymmetric carbon atom, or to a carbon atom situated at a distance from the asymmetric center. Thus, in the case where X is the group —COOH, it was found that it furnished a dextrorotatory contribution in the series of disubstituted propionic acids, whereas in the configurationally related disubstituted acetic acids the contributions of the carboxyls were levorotatory. Should this effect of distance be found general, irrespective of the nature of the group X, it would furnish a basis for a method for correlating the configurations of substances of the above type having $n = 0$ to those having $n = 1$, in cases where the correlation cannot be established by direct chemical means.

These considerations led us to undertake a systematic study of the effect on the optical rotation of the distance from the asymmetric carbon atom of groups possessing a near ultra-violet absorption

band.   The present communication contains a report on the preparation of two configurationally related nitriles, namely

$$CH_3\text{—}\underset{\underset{H}{|}}{\overset{\overset{OCH_3}{|}}{C}}\text{—}CN \quad \text{and} \quad CH_3\text{—}\underset{\underset{H}{|}}{\overset{\overset{OCH_3}{|}}{C}}\text{—}CH_2\text{—}CN$$
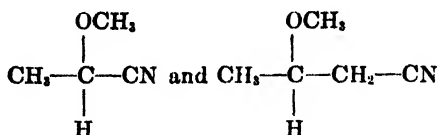
TABLE I

Maximum Rotations of Derivatives of α-Hydroxypropionic and of β-Hydroxybutyric Acids

$CH_3\text{—}\overset{OH}{\underset{H}{C}}\text{—}COOH$		$CH_3\text{—}\overset{OH}{\underset{H}{C}}\text{—}COOC_2H_5$
+4.0		−12.2
$CH_3\text{—}\overset{OCH_3}{\underset{H}{C}}\text{—}COOH$	$CH_3\text{—}\overset{OCH_3}{\underset{H}{C}}\text{—}CN$	$CH_3\text{—}\overset{OCH_3}{\underset{H}{C}}\text{—}COOC_2H_5$
−80.7	−117 5	−119.6
$CH_3\text{—}\overset{OH}{\underset{H}{C}}\text{—}CH_2COOH$		$CH_3\text{—}\overset{OH}{\underset{H}{C}}\text{—}CH_2COOC_2H_5$
+25.0		+33.0
$CH_3\text{—}\overset{OCH_3}{\underset{H}{C}}\text{—}CH_2COOH$	$CH_3\text{—}\overset{OCH_3}{\underset{H}{C}}\text{—}CH_2CN$	$CH_3\text{—}\overset{OCH_3}{\underset{H}{C}}\text{—}CH_2COOC_2H_5$
+13.7	−13.9	+10.6

the first being derived from lactic acid and the second from β-hydroxybutyric acid.   The rotations of the intermediate derivatives and of the nitriles are recorded in Table I in which the rotations of the corresponding ethyl esters are also given.

From Table I it may be seen that the two methoxy acids rotate in opposite directions. Likewise, the esters rotate in opposite directions, and it is quite probable that the contributions of the carboxyl groups as well as the —$COOC_2H_5$ groups are of opposite sign in the methoxy lactic and methoxy-β-butyric acids. In the case of the nitriles the direction of rotation is identical in the two nitriles. However, the values of the rotation of the configurationally related α-methoxypropionitrile and of β-methoxybutyronitrile are of entirely different order of magnitude. This difference in the values of the rotation may be explained on the assumption that in the case of the α-methoxypropionitrile the two contributions—that of the —CN group and that of the rest of the molecule—are of the same sign, whereas in the case of the second nitrile the two contributions are of opposite sign. A study of the dispersion curves of the two substances should give a definite answer to the questions here raised. It is planned to undertake the study of the dispersion curves of these substances in this laboratory.

<div align="center">EXPERIMENTAL</div>

Levo-Ethyl Ester of α-Methoxypropionic Acid,

$$\underset{\displaystyle |}{OCH_3}$$

CH_3—CH—$COOC_2H_5$ 40 gm. of ethyl lactate. $[M]_D^{25} = -7.55°$ (homogeneous), were dissolved in 200 cc. of methyl iodide, and 240 gm. of silver oxide were added in 10 gm. quantities at 15 minute intervals with stirring,[1] keeping the temperature at 50°. The silver salts were filtered off and extracted with ether. The combined filtrate and ether extract was evaporated and then distilled. B. p. 142° at 760 mm. Yield 37 gm.

$$[\alpha]_D^{20} = \frac{-53.8°}{1 \times 0.9551} = -56.3°; \quad [M]_D^{20} = -74.4° \text{ (homogeneous)}$$

$$\text{Maximum } [M]_D^{20} = -119.6° \qquad ``$$

4.416 mg. substance: 8 780 mg. CO_2 and 3.540 mg. H_2O.
<div align="center">$C_6H_{12}O_2$. Calculated. C 54.5, H 9.2
Found.   `` 54.2, `` 9.0</div>

$$\underset{\displaystyle |}{OCH_3}$$

Levo-α-Methoxypropionic Acid, CH_3—CH—$COOH$ —37 gm.

[1] Hibbert, H., Tipson, R. S., and Brauns, F., *Canad. J. Research*, **4**, 221 (1931).

of ethyl ester of α-methoxypropionic acid, $[M]_D^{20} = -74.3°$ (homogeneous), were hydrolyzed by refluxing for 1 hour with 30 gm. of potassium hydroxide in 80 per cent alcohol. The alcohol was evaporated and the salt acidified with hydrochloric acid. The organic acid was extracted in a continuous extractor. B. p. 92° at 15 mm. Yield 20 gm.

$$[\alpha]_D^{25} = \frac{-52.6°}{1 \times 1.091} = -48.2°; \quad [M]_D^{25} = -50.2° \text{ (homogeneous)}$$

$$\text{Maximum } [M]_D^{25} = -80.7° \qquad \text{``}$$

4.505 mg. substance: 7.720 mg. CO_2 and 3.190 mg. H_2O
$C_4H_8O_3$. Calculated. C 46.1, H 7.7
                    Found.   " 46.7, " 7.9

$$\overset{\displaystyle OCH_3}{\underset{\displaystyle |}{}}$$

Levo-α-Methoxypropionic Nitrile, $CH_3—CH—CN$ —40 gm. of α-methoxypropionic acid, $[M]_D^{25} = -50.1°$ (homogeneous), were refluxed with 100 gm. of thionyl chloride during 30 minutes and then fractionated. The acid chloride was not further purified, but was dropped into an excess of aqueous ammonia at 0°. The amide was extracted with ether in a continuous extractor. The ether was evaporated and the solid amide was heated in a distilling flask with an excess of phosphorus pentoxide. The product obtained was redistilled. B. p. 115° at 735 mm. Yield 8 gm.

$$[\alpha]_D^{20} = \frac{-76.7°}{1 \times 0.893} = -85.9°; \quad [M]_D^{20} = -73.1° \text{ (homogeneous)}$$

$$\text{Maximum } [M]_D^{20} = -117.5° \qquad \text{``}$$

5.135 mg. substance: 10.615 mg. CO_2 and 3.890 mg. H_2O.
5.570 "   "   : 0.857 cc. N_2 at 25° and 736 mm.
       C_4H_7ON. Calculated. C 56.4, H 8.3, N 16.5
                          Found.   " 56.4, " 8.5, " 17.0

Levo-β-Hydroxybutyric Acid ($CH_3 \cdot CHOH \cdot CH_2 \cdot COOH$) —600 gm. of inactive β-hydroxybutyric acid were dissolved in 2500 cc. of water and heated on a steam bath. Quinine was added and the product crystallized by cooling to −5°. After three recrystallizations the precipitate was decomposed by shaking with 2 mols of potassium hydroxide in 1 liter of water. It was extracted with chloroform to free it from quinine and then the aqueous layer was

evaporated to 1 liter. A solution of 100 gm. of sulfuric acid in 750 cc. of water was added and the acid was extracted with ether with a continuous extractor.

$$[\alpha]_D^{25} = \frac{-0.90° \times 100}{1 \times 8.84} = -10.2°; \quad [M]_D^{25} = -10.5° \text{ (in water)}$$

$$\text{Maximum } [M]_D^{25} = -25.0° \quad " \quad "$$

Levo-Ethyl Ester of β-Hydroxybutyric Acid ($CH_3 \cdot CHOH \cdot CH_2 \cdot COOC_2H_5$)—80 gm. of β-hydroxybutyric acid, $[M]_D^{25} = -10.5°$ (in water), were dissolved in 100 cc. of absolute alcohol and 5 cc. of concentrated sulfuric acid were added. The product was heated on a steam bath during 1 hour. Most of the alcohol was evaporated and then a paste of potassium carbonate was added. The ester was extracted with ether, dried, and distilled. B. p. 74° at 12 mm.

$$[\alpha]_D^{25} = \frac{-10.6°}{1 \times 1.009} = -10.51°; \quad [M]_D^{25} = -13.88° \text{ (homogeneous)}$$

$$\text{Maximum } [M]_D^{25} = -33.0° \quad "$$

3.900 mg. substance: 7.745 mg. CO_2 and 3.220 mg. H_2O.

         $C_6H_{12}O_3$ Calculated.   C 54.5, H 9.2
                    Found.   " 54.2, " 9.2

Levo-Ethyl Ester of β-Methoxybutyric Acid,

$$\overset{\displaystyle OCH_3}{\underset{\displaystyle |}{CH_3 - CH - CH_2 \cdot COOC_2H_5}}$$

—50 gm. of ethyl ester of β-hydroxybutyric acid, $[M]_D^{25} = -13.86°$ (homogeneous), were dissolved in 310 gm. of methyl iodide and 260 gm. of silver oxide were added in 10 gm. portions at 15 minute intervals with stirring, the temperature being maintained at 40–50°. The product was extracted from the silver salts with ether and then distilled. B. p. 165° at 760 mm. Yield 50 gm.

$$[\alpha]_D^{25} = \frac{-1.95°}{1 \times 0.962} = -2.03°; \quad [M]_D^{25} = -2.96° \text{ (homogeneous)}$$

$$\text{Maximum } [M]_D^{25} = -10.6° \quad "$$

4.941 mg. substance: 10.250 mg. CO_2 and 4.050 mg. H_2O.

         $C_7H_{14}O_3$.   Calculated.   C 57.5, H 9.7
                    Found.   " 56.6, " 9.2

$$OCH_3$$
$$|$$

Levo-β-Methoxybutyric Acid, CH_3—CH—$CH_2 \cdot COOH$ — 50 gm. of ethyl ester of β-methoxybutyric acid were hydrolyzed by 30 gm. of potassium hydroxide in 80 per cent alcohol. After distilling off the alcohol, the product was acidified with hydrochloric acid and extracted with ether with a continuous extractor. B. p. 115° at 15 mm. Yield 35 gm.

$$[\alpha]_D^{25} = \frac{-3.44°}{1 \times 1.060} = -3.25°; \quad [M]_D^{25} = -3.83° \text{ (homogeneous)}$$

$$\text{Maximum } [M]_D^{25} = -13.7° \qquad \text{“}$$

3.880 mg. substance: 7.115 mg. CO_2 and 2.830 mg. H_2O
$$C_5H_{10}O_2. \quad \text{Calculated.} \quad C\ 50.8,\ H\ 8.5$$
$$\text{Found.} \qquad \text{“ } 50.0,\text{ “ } 8.2$$

$$OCH_3$$
$$|$$

Dextro-β-Methoxybutyric Nitrile, CH_3—CH—$CH_2 \cdot CN$ — 35 gm. of β-methoxybutyric acid, $[M]_D^{25} = -17.3°$ (homogeneous), were converted into the amide through the acid chloride, as described for α-methoxypropionic nitrile. The amide was distilled with an excess of phosphorus anhydride, and then redistilled. B. p. 129° at 740 mm.

$$[\alpha]_D^{25} = \frac{+3.40°}{1 \times 0.880} = +3.9°; \quad [M]_D^{25} = +3.8° \text{ (homogeneous)}$$

$$\text{Maximum } [M]_D^{25} = +13.9° \qquad \text{“}$$

3.695 mg. substance: 8.195 mg. CO_2 and 2.885 mg. H_2O
5.695   “   “   : 0.719 cc. N at 28° and 757 mm.
$$C_5H_9ON. \quad \text{Calculated.} \quad C\ 60.6,\ H\ 9.2,\ N\ 14.1$$
$$\text{Found.} \qquad \text{“ } 60.5,\text{ “ } 8.8,\text{ “ } 14.2$$

A DIETARY FACTOR CONCERNED WITH CARBOHY-
DRATE METABOLISM*

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A metabolic abnormality in rats on a fat-deficient diet in which
carbohydrate is converted into fat to a degree not found in normal
animals has been described in previous papers (1-3). If this ab-
normality is caused by a deficiency in their restricted diet of some
factor concerned with carbohydrate metabolism, it should be
possible to correct or prevent this condition by supplying the de-
ficient factor. The known vitamins and the essential amino acids
are not effective in this respect (1-3). Neither is linoleic acid
effective, even when fed in large amounts and over a relatively long
period of time (2), although it is curative for Burr's fat deficiency
disease (4). However, when certain fats are fed to rats whose
carbohydrate metabolism is abnormal, a more nearly normal con-
dition is attained (1). This finding has recently been confirmed
by Burr and Beber (5).

A fat which apparently contains this corrective factor is lard
(1). In the present paper we shall show by results obtained with
several lard preparations that the effectiveness of a fat in reestab-
lishing normal carbohydrate metabolism is greater when larger
amounts of the fat are fed to the abnormal rats; that the deficiency
of a factor and not the need of fat for the purpose of catabolism
by the rats maintained on a fat-deficient diet is the cause of the
abnormal conversion of carbohydrate to fat; that no metabolic
abnormality develops in rats with which the preventive method of

vitamin testing has been used; and that the dietary factor is apparently of acid character, and is resistant to saponification.

EXPERIMENTAL

Diet—The diet consisted of sucrose, 84.5 per cent; alcohol- and ether-extracted casein (containing about 0.04 per cent fat), 12 per cent; inorganic salt mixture (6), 3.5 per cent; and, in addition, daily doses of ether-extracted brewers' yeast (Harris), 0.5 to 0.6 gm.; carotene (Mead Johnson), 0.025 mg.; ergosterol (Mead Johnson), irradiated, 0.1 mg.[1]   Most of the rats used in the present series of experiments were started on this fat-deficient diet at weaning or 1 to 2 weeks later.

Preparations. *"Liquid Fat" Fraction of Lard and "Solid Fat" Fraction of Lard*—1 part by weight of lard (Swift's Silver Leaf brand) was crystallized from 2 volumes of absolute alcohol.   The crystals were further purified by one recrystallization.   The alcohol was expelled from the first mother liquor and from the crystals by evaporation *in vacuo* at room temperature, then for a brief period at 80°.   The mother liquor yields the liquid fat fraction, and the crystals the solid fat fraction of the lard.

Saponifiable Portion of Liquid Fat Fraction of Lard—15 gm. of the liquid fat fraction of lard were saponified by means of 6 gm. of KOH in 30 cc. of 95 per cent alcohol, by heating the mixture on the steam bath just to boiling, followed by immediate dilution with water and cooling.   The unsaponified compounds were removed and the saponifiable material recovered in the usual way.

Ethyl Stearate (Eastman Kodak Company)

Procedure for Detecting Abnormal Carbohydrate Metabolism— The test for abnormal carbohydrate metabolism used in the work described in this paper, as in preceding ones (1-3), is the attainment of respiratory quotients above 1.00 during the assimilation of carbohydrate.

The rats were kept for at least 3 to 4 days in a constant temperature (28°) metabolism room (7) prior to the determinations, and

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[1] 100 mg. of ergosterol after 15 minutes irradiation in a thin layer of absolute ether solution and 25 mg. of carotene are together made up to 1 liter with absolute ether.   1 cc. of this solution was evaporated on each daily individual portion of yeast, then sufficient water added to make a thin paste of the yeast.

most of the animals were kept in this room throughout the period of restricted diet. After a number of months on the fat-deficient diet, a rat was fasted for approximately 18 hours, after which the carbohydrate (dextrin) test meal (2, 3, 8) was fed, and respiratory gas exchange determinations made hourly during the 6 hours after this feeding.[2] Under these conditions, respiratory quotients greater than 1.00 are indicative of abnormal fat formation from carbohydrate.

Procedure Used in Testing a Preparation for Its Activity in Restoring an Abnormal Carbohydrate Metabolism to a More Nearly Normal One, or in Preventing Onset of Abnormal Carbohydrate Metabolism—If the respiratory quotient of a rat was found to be above 1.04 in one or more of the five hourly determinations after the carbohydrate test meal, the rat was fed, in addition to its regular diet, a weighed amount of one of the preparations described above for 3 successive days, fasted on the 4th day, and another series of respiratory quotient determinations was made on the 5th day. In case the respiratory quotients were found to be still high, the procedure was repeated until a decided effect in lowering the respiratory quotients was observed. 1 or 2 weeks later another series of determinations was made to learn whether the dosing was still effective, then again at intervals until abnormal quotients were again obtained. In the series of experiments in which large doses of the liquid fat fraction and ethyl stearate were fed, the schedule for the metabolism tests was as follows: the first test, just before feeding the preparation; the second test, after feeding the liquid fat fraction and ethyl stearate for 3 successive days; the third test, after feeding the same preparation for another period of 3 days following the second test; the fourth test, 1 week later; the fifth test, after the further lapse of 2 to 3 weeks, and repeatedly thereafter.

For the preventive method, rats from two litters were equally distributed at weaning among three groups: the first group received daily in addition to the fat-deficient diet 6.67 mg. per rat of the liquid fat fraction; the second, the same weight of the solid

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[2] The closed circuit respiratory calorimeter previously described (8) was used for this purpose. The only change has been the substitution of a small rotary gear pump and appropriate form of scrubber for the piston type of pump formerly used.

fat fraction; while the third received no fat at all.   At the end of 4 months and again 1 week later the respiratory quotients of carbohydrate assimilation were determined by the method described above.

Results

As in the preceding paper (3) of this series, several additional criteria of the degree of fat formation are obtained by plotting graphs of the hourly respiratory quotients of the period of most active carbohydrate metabolism.   One of these is the area of the curves above the line R. Q. = 1.00, areas that represent the duration as well as the intensity of fat formation.   Another criterion is the amount by which the areas of the abnormal curves exceed that of the average *normal* carbohydrate assimilatory respiratory quotient curve obtained under the same conditions (3, 8).   These graphs were plotted 0.1 unit R. Q. = 1 inch, 1 hour = 1 inch, and the areas, determined by means of a planimeter, are expressed in sq. inches.

Liquid and Solid Fat Fractions, Curative Method—An average of 0.7 gm. of the liquid fat fraction of lard per 100 gm. of body weight was sufficient to restore carbohydrate metabolism to nearly normal in a group of twelve rats (Table I), while 7.7 gm. per 100 gm. of body weight of the solid fat fraction were required (Table I) for another group of thirteen rats.   Furthermore, this small amount of the liquid fat fraction kept the carbohydrate assimilatory respiratory quotients near normal for 25 days without further dosing before the reappearance of the metabolic abnormality, while 10 times this amount of the solid fat fraction was effective for 18 days only.   A quantitative difference of this magnitude in the relative effectiveness of the two fractions of the same fat seems to us to be of significance, first, by helping to eliminate the question of the fat *per se* as being the effective agent, and secondly, by demonstrating a method by which this carbohydrate factor of lard may be concentrated.

Effect of Liquid Fat Fraction and of Ethyl Stearate in Relatively Large Amounts—In obtaining the results described in the preceding paragraph, small amounts of these two preparations from lard were used in repeated doses.   In order to test the effect of relatively large amounts of the liquid fat fraction on the abnormal

carbohydrate quotients, seven abnormal rats were given a total of 6 gm. of this fraction per 100 gm. of body weight over a period of 8 days. This amount was successful in quickly lowering the respira-

TABLE I

Respiratory Metabolism of Rats on a Fat-Deficient Diet without and with Feeding of Liquid Fat and Solid Fat Fractions and Saponifiable Portion of Liquid Fat of Lard

| | Average maximum R Q | Average area of R Q. curve | | Average metabolic rate, sq. m per 24 hrs |
		Above normal	Above 1 00	
Curative treatment with liquid fat fraction, with 12 rats, average total dose 0.7 gm. per 100 gm. body weight				
		sq. in.	*sq. in.*	*calories*
Before dosing	1 11	5 72	1 49	865
After "	1 01	2 71	0 12	877
Curative treatment with solid fat fraction, with 13 rats, average total dose 7.7 gm. per 100 gm. body weight				
Before dosing	1 06	5 39	1 19	840
After "	1 02	3 34	0 21	885
Curative treatment with saponifiable portion of liquid fat, with 13 rats, average total dose 2.37 gm. per 100 gm. body weight				
Before dosing	1 08	5 43	1 38	778
After "	1 03	4 08	0 26	803
Preventive treatment with solid fat (6 rats) and liquid fat fractions (4 rats). daily dose 6 67 mg. per rat for 4 months, compared with a control group (5 rats) that received only the fat-deficient diet				
Liquid fat	0 99	2 30	0 09	830
Solid "	0 97	1 05	0 09	787
Controls (no fat)	1 05	4 90	1 16	695

tory quotient curves of carbohydrate assimilation to the point at which, on the average, practically no indication of the transformation of carbohydrate into fat could be detected (Table II).

In contrast to this effect were the results (Table II) obtained with another group of eight abnormal rats fed ethyl stearate simi-

larly to those fed the liquid fat fraction of lard.   It is seen that no effect was obtained in reducing the height to which the respiratory quotient curve attains, nor in reducing the area enclosed by the curves above the line R. Q. = 1.00.   The results obtained with this group of rats are a further indication that the abnormal formation of fat from carbohydrate takes place, not because of a need of fat

TABLE II

Respiratory Metabolism of Abnormal Rats before and following Feeding of the Liquid Fat Fraction of Lard and Ethyl Stearate.   Curative Method.   Total Dose of Each, 3 Gm. per 100 Gm. Body Weight

Fat fed	No of rats	After starting to dose	Average maximum R Q	Average area of R Q curve		Average metabolic rate, sq m per 24 hrs
				Above normal	Above 1 00	
		days		*sq in*	*sq in*	*calories*
Liquid fat fraction	7	0	1 07	4 94	1 25	823
		4	1 04	3 69	0 53	845
		8	1 04	3 53	0 55	834
		15	1 02	2 99	0 48	733
		29	0 99	2 51	0 15	781
Ethyl stearate	8	0	1 09	5 90	1 75	728
		4	1 07	5 17	1 33	787
		8	1 08	5 28	1 46	766
		15	1 09	5 29	1 37	725
		29	1 07	5 67	1 58	764
Ethyl stearate followed by liquid fat fraction	7	0	1 08	4 43	1 09	846
		4	1 09	5 30	1 58	783
		8	1 07	5 40	1 20	784
		12	1 07	5 44	1 63	800
		16	1 08	5 90	1 82	860
		23	1 04	3 91	0 50	838
		37	1 04	4 39	0 70	780

per se for the purposes of catabolism but because of the deficiency of a metabolic factor in food fat.

For the third group of abnormal rats the feeding of the liquid fat fraction followed the 8 days feeding of ethyl stearate.   As in the case of the two former groups, 6 gm. of the two preparations were given in the two successive 8 day periods.   It was found that for this group (Table II) a lowering of the respiratory quotient curve was not immediately brought about by the liquid fat, as before,

but a delay was caused apparently by the stearate feeding that preceded it.   As this phenomenon may be of some significance in the study of the mechanism of the deficiency symptom, it will be further investigated.

Preventive Method, Liquid and Solid Fat Fractions—Since the prevention of the appearance of the symptom of a deficiency disease is of equal significance to its cure by a dietary factor, the liquid fat fraction of lard was fed along with the fat-deficient diet over 4 months time to a group of young rats beginning shortly after weaning, and an equal amount of the solid fat fraction to another
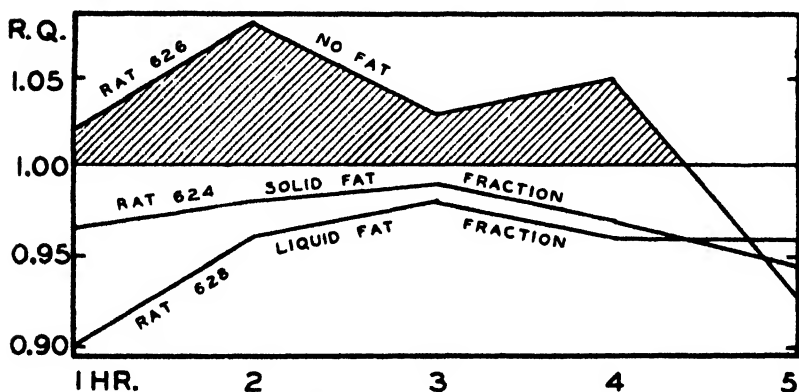
FIG. 1. Graphs illustrating the effect of preventive or prophylactic treatment with the solid fat and liquid fat fractions of lard on the carbohydrate assimilatory respiratory quotients of individual rats.   The summary of the respiratory metabolism when the preventive treatment is used is given in Table I.

group.   A minute daily amount (6.67 mg.) of either fraction was sufficient to prevent the appearance of the metabolic abnormality, while the control group, litter mates of the other two groups, receiving no fat, exhibited a pronounced abnormality (Table I and Fig. 1).   On the other hand, the difference in the control group from the other groups as regards growth was only moderately great, the average weights at the end of the 4 months being 94 gm., 105 gm., and 119 gm. respectively, as compared with about 235 gm. for average normal rats at 5 months of age.   The average length from nose to anus for the three groups was 17.9 cm. for the control group, as compared with 17.8 cm. for the group receiving

the liquid fat fraction and 18.6 cm. for the group receiving the solid fat fraction.   The corresponding length for 5 months old normal rats is about 20 cm.   The average percentage emaciation (2, 9) for the three groups was 76, 66, and 66 per cent respectively.   It is seen that although the groups that were fed small amounts of these fat preparations daily during 4 of the 5 months of their life and growth showed nearly normal carbohydrate assimilatory respiratory quotients, they were almost as stunted and emaciated as the control group that received only a fat-deficient diet.   Stunting and emaciation therefore cannot be considered the cause of the abnormal quotients given by the control group of rats.

Saponifiable Fat—That the saponifiable portion of the liquid fat fraction of lard after a thorough extraction of the unsaponified fraction is active (Table I), is significant for several reasons—it clearly points to the acid character of the dietary factor as present in lard, and it demonstrates the resistance of the factor to the process of saponification.   Although a larger amount of this saponifiable portion of the liquid fat fraction was required to reduce the metabolic abnormality than of the liquid fat fraction before saponification, the average time that the effect of the saponifiable fraction lasted was 42 days as compared with 25 days for the fat before saponification.   Thus, there is no indication that the alkaline liquid as used in the saponification procedure described above has even partially destroyed the carbohydrate factor.

SUMMARY

A metabolic abnormality in rats on a fat-deficient diet in which carbohydrate is converted into fat to a degree not found in normal animals is corrected by supplying any of several lard preparations. One of these, the liquid fat fraction of lard, is found to be more than 10 times as active in this respect as the solid fat fraction of lard. While relatively large amounts of the liquid fat fraction of lard were rapidly effective in correcting the metabolic abnormality, the same amount of ethyl stearate was inactive.   However, the liquid fat fraction of lard is not as rapidly effective if ethyl stearate is fed along with the fat-deficient diet for a few days prior to the liquid fat feeding.

Small daily amounts (6.67 mg.) of the liquid fat or of the solid

fat fraction of lard prevent the appearance of the metabolic abnormality, although stunting and emaciation are present to nearly the same degree as in the control group that received the fat-deficient diet only.

This abnormal formation of fat from carbohydrate by the rat is not brought about because of a need of fat for catabolism, but because of a deficiency of some metabolic factor in food fat.

The saponifiable fraction of the liquid fat of lard is active, thus indicating both the acid nature of the dietary factor and its resistance to saponification.

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STUDIES ON PURINE METABOLISM

II. THE FATE OF GUANINE IN THE ORGANISM OF THE DOG

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The results of the investigations of the senior author and his coworkers on the metabolism of pyrimidine compounds in the dog encouraged us to investigate the physiological behavior of purine substances in the same animal. These constituents of the nucleic acid molecule have acquired in recent years an increased interest, in view of the findings in various fields of investigation, dealing with the rôle of adenylpyrophosphoric acid in the muscle, the function of an adenine nucleotide as the coenzyme of alcoholic fermentation, and the action of adenine compounds on the vascular system.

As in the case of the pyrimidines, we decided to investigate first the metabolism of the purine bases before proceeding to the more complex constituents of the nucleic acid molecule. We were particularly interested in ascertaining whether these substances undergo in the dog a breakdown beyond the allantoin stage. The experiments of Frank and Schittenhelm (1) and of Severin (2) suggest that in man uric acid may be further oxidized to urea. The findings of Shimoda (3) point to a similar behavior in the rabbit. Quite recently, Chrometzka (4) reported that allantoin, when fed to man, is partly converted into urea.

The experiments to be discussed were conducted in an endeavor to study the metabolism of guanine and adenine in the dog. There are only two investigations on record dealing with the physiological behavior of guanine in the dog. Stadthagen (5) fed 6 gm. of guanine to a dog, and observed no changes in the uric acid or purine output. Ewald (6) injected the base in piperazine solu-

tion intravenously.   His data are meaningless in view of the leucocytosis which the injected material produced.   Adenine was fed to dogs by Minkowski (7) and found to be toxic.

	Day	N intake	Output in urine		
			Total N	Urea N	Allantoin N
		gm.	gm.	gm.	gm
Experiment 1. Dog A, weight 9 5 kilos	1	4 50	3.95	3 25	0.10
	2	4 50	3 98	3 20	0 11
	3	5 18	3 86	3 13	0.11*
	4	4 50	4 32	3 40	0 34
	5	4 50	3.90	3 15	0 12
	6	4 50	3 88	3 12	0 13
Experiment 2. Dog A, weight 9 5 kilos	1	4 56	3 92	3 17	0.10
	2	4.56	3.98	3 20	0 11
	3	4 56	3 95	3 13	0.11
	4	5 25	3 93	3.13	0 10†
	5	4 56	4 23	3 30	0.20
	6	4 56	4 02	3 09	0 19
	7	4 56	3 82	3 11	0 10
	8	4 56	4 00	3.15	0 10
Experiment 3. Dog B, weight 10.4 kilos	1	5 60	4 98	4.12	0.13
	2	5 60	5 09	4 20	0 12
	3	5 60	4 84	4 00	0 12
	4	6 28	4 88	4 07	0.12*
	5	5 60	5 36	4 27	0 36
	6	5 60	4 98	4 10	0.14
Experiment 4. Dog B, weight 10.4 kilos	7	5 60	4 90	4 00	0.12
	8	5 60	5 03	4 11	0 12
	9	6 29	4 89	4 05	0 12†
	10	5 60	5 32	4.22	0 27
	11	5 60	4 99	4 05	0 16
	12	5 60	4 93	4 07	0.13

* 2.0 gm. of guanine hydrochloride were fed with the food.   N = 0.68 gm.

† 1.5 gm. of guanine were fed with the food.   N = 0.69 gm.

EXPERIMENTAL

The technique of the feeding experiments has been described in a previous paper (8).   The following analytical methods were used: total N, Kjeldahl; urea, Allen and Luck (9); ammonia, the

Folin and Bell permutit method (10); allantoin, Allen and Cerecedo (11); inorganic sulfur, precipitation with benzidine according to Rosenheim and Drummond (12).   The purity of the compounds fed was checked by analysis.

Experiments with Guanine—Guanine was fed as the free base and as the hydrochloride.   An examination of the data in Table I shows that the substance is better absorbed when given in the form of the hydrochloride.   This confirms the observations of previous workers.   We find that the hydrochloride when fed in doses of 2.0 gm. is metabolized to the extent of 70 to 80 per cent. One-third of the nitrogen present in the compound is accounted for by the increase in the urea output on the day after feeding. The rise in allantoin corresponds to another third.   The amino group in guanine represents one-fifth of the nitrogen, so that more urea has been formed than should be expected from the conversion of this amino group into urea.

In the case of the free base (Experiments 2 and 4) we find that it is metabolized to a lesser extent than its hydrochloride.   This we believe to be due to the lower solubility of the substance.   Otherwise, the results are practically the same as those obtained with the hydrochloride.

Ammonia and inorganic sulfates were determined every day.   It was found that these two urinary constituents remained practically unchanged on the day following the ingestion of guanine and its hydrochloride.   Inasmuch as these data did not reveal any significant facts, they have not been included in Table 1.

Several attempts were made to determine the metabolism of adenine.   In every case the compound was found to be toxic. Even amounts as small as 1.3 gm. will cause vomiting in the dog.

DISCUSSION

We believe that our experiments with guanine indicate that in the dog there might be a further breakdown of the purine molecule beyond the allantoin stage to yield urea.   It is possible that one of the intermediate substances in the path of this breakdown is oxaluric acid.   The possibility that this compound may be a link connecting the catabolic breakdown of purines and pyrimidines in the animal body has been considered by Cerecedo (13). Chrometzka (4) reports one experiment in which, after 20 gm. of nucleic acid was fed to man, a substance was excreted in the urine

which, on hydrolysis with acid, gave rise to oxalic acid. This he believes to be either oxaluric acid or a similar compound. Our experiments with adenine confirm the observations of Minkowski as to the toxicity of the substance. We are reminded of the experiments of Jones and Austrian (14) and Amberg and Jones (15), who were unable to find adenase, the enzyme which converts adenine into hypoxanthine, in the tissues of the dog. In connection with the recent observations of Bennet and Drury (16) and others on the action of adenine compounds on the heart, it may be of interest to point out that Minkowski seems to have been the first investigator to observe such effects. He injected 0.5 gm. of adenine subcutaneously into a dog. He described the action of the compound as follows: "Wenige Minuten später fällt die sehr erregte Herzaction auf. Der Herzstoss ist sehr verstärkt und führt zu solchen Erschütterungen des ganzen Käfigs, dass man die lauten Schläge im Zimmer hören kann."

SUMMARY

Experiments are described in which guanine and adenine were fed to dogs. Evidence was obtained that guanine, when fed in small amounts, either as the free base or as the hydrochloride, is partly converted into allantoin and partly broken down to yield urea. Adenine, when fed in amounts as small as 1.3 gm., was found to be toxic.

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DERIVATIVES OF MONOACETONE XYLOSE

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In connection with the general problem of the synthesis and behavior of the phospho sugars, it was desired to prepare 3-phospho xylose   For this purpose, monoacetone xylose seemed to be the obvious starting material inasmuch as Haworth and Porter[1] had shown it to be 1,2-monoacetone xylofuranose, thus having only positions (3) and (5) unsubstituted.   If, as in the case of monoacetone glucose, preferential substitution of the primary alcoholic group could be achieved, then mono derivatives in positions (3) and (5) should be accessible.

By reacting with a single equivalent of the substituent it was possible to obtain monosubstituted acetyl, benzoyl, and p-tolylsulfo monoacetone xyloses.   In order to establish the position of the acyl group in each, the acetyl and benzoyl derivatives were tolylsulfonated; on the other hand the tolylsulfo compound was acetylated in one case and benzoylated in the other.   Thus two acetyl p-tolylsulfo and two benzoyl p-tolylsulfo monoacetone xyloses were secured. In each pair the two substances were not identical, but isomeric, so that the original monoacyl derivatives apparently all had the substituent in the same position.   By analogy with monoacetone glucose this was expected to be position (5).   For verification of this assumption the various tolylsulfo derivatives were heated with sodium iodide in acetone, a procedure shown by Oldham and Rutherford[2] to produce ready removal of a tolylsulfo radicle only when it is attached to a primary alcoholic group.   The results of the experiments were in agreement with the assumption made above.   Thus in the three

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[1] Haworth, W. N., and Porter, C. R., *J. Chem. Soc.*, 611 (1928).

[2] Oldham, J. W. H., and Rutherford, J. K., *J. Am. Chem. Soc.*, **54**, 366 (1932).

substances derived by monoacylation of monoacetone xylose, the acyl groups apparently occupy position (5).

Results which will be reported in a later paper led us to question the homogeneity of the 5-benzoyl monoacetone xylose, and the substance was therefore acetylated in one case and p-tolylsulfonated in another. The latter procedure gave only a 60 per cent yield of the pure tolylsulfo benzoyl monoacetone derivative but the acetylation gave 91 per cent of crude crystalline product and 84 per cent of carefully purified acetyl benzoyl derivative. The benzoyl monoacetone xylose may therefore be regarded as being largely homogeneous. The orthobenzoate structure, with the benzoyl linked to both positions (3) and (5) was also considered. The light absorption of the product was therefore measured in the ultra-violet region of the spectrum and was compared with that of a product which was almost certainly 3-methyl-5-benzoyl monoacetone xylose. It was found that these two compounds had identical molecular absorptions in the region characteristic of the C_6H_5CO group so that the orthobenzoate structure is excluded. For the moment, therefore, there is no evidence against the view that this substance has the structure, 5-benzoyl monoacetone xylose.

Additional attempts at partial substitution of the monoacetone xylose led us to prepare the 5-iodo derivative, and from this the 5-nitrate. A nicely crystalline monobenzylcarboxy monoacetone xylose, presumably substituted in position (5), was also prepared. It is very readily hydrolyzed by dilute alkali but not by dilute acid, and may prove to be a useful product in certain cases. We plan to prepare other benzylcarboxy derivatives of the sugars and to study their properties and possible uses as intermediates in sugar syntheses.

<center>EXPERIMENTAL</center>

Diacetone Xylose—To 2 liters of u.s.p. acetone were added, in order, 10 cc. of concentrated sulfuric acid, 200 gm. of powdered anhydrous copper sulfate, and 100 gm. of xylose. After shaking in the machine for 24 hours, the copper sulfate and unchanged xylose were filtered off, 25 cc. of water were added, and the solution was neutralized by shaking with powdered calcium hydroxide. After filtering, the solution was concentrated to a thick syrup. For preparation of monoacetone xylose this syrup may be used directly.

For crystallization, however, it was dissolved in 50 cc. of methyl alcohol, and after cooling in the ice box, water was added almost to turbidity (25 cc.). On standing, the diacetone crystallized. Further crops were obtained by concentrating and crystallizing from 50 per cent methyl alcohol. The total yield was 100 to 110 gm. of a slightly sticky, crude product. One more crystallization from about 50 per cent methyl alcohol gives a colorless crystalline material in a yield of 90 to 95 gm. The diacetone xylose may be recrystallized from aqueous methyl alcohol, or from pentane, but the product crystallized from the latter solvent is apparently never entirely pure. After three crystallizations from pentane and two from aqueous methyl alcohol, the melting point of the material was 44–45°. The rotation, in water, was $[\alpha]_D^{22} = \dfrac{+0.52° \times 100}{2 \times 2.0} = +13.0°$, and in chloroform was $[\alpha]_D^{22} = \dfrac{+0.24° \times 100}{2 \times 2.0} = +6.0°$. The composition agreed with that of a diacetone pentose.

5.020 mg. substance: 10.550 mg. CO_2 and 3.525 mg. H_2O
$C_{11}H_{18}O_5$.   Calculated.   C 57.37, H 7.88
230.1   Found.   " 57.32, " 7.87

The product is very soluble in all the common organic solvents except pentane in which it is only moderately soluble in the cold. It is quite soluble in warm water and, on cooling, crystallizes out very slowly.

Monoacetone Xylose—Crude, syrupy diacetone xylose, prepared as above, was converted to the monoacetone by the procedure of Svanberg and Sjöberg.[3] A completely colorless product was obtained by hydrolyzing the crystalline diacetone xylose with 0.2 N sulfuric acid in a similar fashion. Prepared by either method the substance crystallized easily after distillation but its recrystallization from a solvent has not been effected.

5-Acetyl Monoacetone Xylose—10 gm. of crystalline monoacetone xylose were dissolved in 25 cc. of dry pyridine, cooled in ice, and stirred mechanically. A solution of 5.5 cc. (1.1 mols) of acetic anhydride in 15 cc. of chloroform was dropped in over a period of about 20 minutes. The mixture was then removed from

[3] Svanberg, O., and Sjöberg, K., *Ber. chem. Ges.*, **56**, 863 (1923).

the bath and allowed to stand overnight at room temperature. 2 cc. of water were added, the mixture was left for $\frac{1}{2}$ hour at room temperature, and was then diluted with ice water. The product was extracted with several portions of chloroform, and the combined extracts were washed with dilute ice-cold, sulfuric acid until faintly acid. After two washings with water the chloroform extracts were dried with sodium sulfate and concentrated under reduced pressure to a thick syrup. This was taken up in ether and cooled. Pentane was added just to turbidity and on seeding and standing the product crystallized. The seed crystals were obtained from a portion of the material which had been allowed to stand for several days in an open dish. The crystalline product was filtered off and recrystallized from ether-pentane in the same manner. The yield was only 2.6 gm. or 21 per cent of the theoretical. The material was dissolved in ether and the solution was concentrated, cooled, and stirred to effect crystallization. The pure product melted at 100–100.5° and had a rotation, in chloroform, of

$$[\alpha]_D^{22} = \frac{+0.92° \times 100}{2 \times 2.0} = +23.0.°$$ The composition agreed with

that of an acetyl monoacetone pentose.

5.175 mg. substance: 9.772 mg. CO_2 and 3.195 mg. H_2O

$C_{10}H_{16}O_6$.   Calculated.   C 51.70, H 6.96

232.1   Found.   " 51.50, " 6.92

The substance is very soluble in water, methyl and ethyl alcohol, ether, acetone, ethyl acetate, and chloroform, fairly soluble in hot heptane and hexane, slightly soluble in hot pentane.

5-Benzoyl Monoacetone Xylose—The procedure for the preparation of this substance was similar to that for the 5-acetyl derivative; 50 gm. of monoacetone xylose in 250 cc. of dry pyridine being treated with 33 cc. (1.1 mols) of benzoyl chloride in 100 cc. of chloroform. The final syrupy product, isolated as before, was taken up in ether and seeded with crystals obtained from a sample which had stood in the air in an open dish. The product crystallized immediately. It was filtered off and additional small amounts were obtained by working up the mother liquors. The yield was 40 to 45 gm. or 55 per cent of the theoretical. The crude material crystallized with greatest ease but the melting point was not sharp. The best product was secured by crystallizing alternately

from ether and from hexane containing a trace of ether. After several crystallizations the melting point was 83.5–84.5° and the rotation, in chloroform, was $[\alpha]_D^{20} = \dfrac{+0.22° \times 100}{2 \times 2.0} = +5.5°$. The composition agreed with that of a benzoyl monoacetone pentose.

5.011 mg. substance: 11.261 mg. CO_2 and 2.750 mg. H_2O
$C_{15}H_{18}O_6$.   Calculated   C 61.20, H 6.17
294.1   Found.   " 61.29, " 6.15

The substance is very soluble in most of the common organic solvents. It is fairly soluble in hot heptane, somewhat soluble in hot hexane, and a little in hot pentane, but almost insoluble in these solvents in the cold. It is slightly soluble in hot and insoluble in cold water. It crystallizes beautifully from ether or pentane and fairly well from hexane.

5-p-Tolylsulfo Monoacetone Xylose—The preparation was similar to that of the 5-acetyl derivative, 50 gm. of monoacetone xylose in 250 cc. of dry pyridine being treated with 55 gm. (1.1 mols) of *p*-toluenesulfonchloride in 100 cc. of chloroform. After 1 hour in the ice bath, the mixture was kept overnight at room temperature. 2 cc. of water were added, and after ½ hour the mixture was diluted with water, the product was extracted with chloroform, and worked up as for the substances above. The syrupy product crystallized immediately upon adding ether. The crystals were filtered off and more product was obtained from the mother liquor. The yield was 55 gm. or 61 per cent of the theoretical. The product was twice recrystallized from ethyl acetate and it then melted at 133–134° and had a rotation, in chloroform, of $[\alpha]_D^{20} = \dfrac{-0.52° \times 100}{2 \times 2.0} = -13.0°$. The composition agreed with that of a tolylsulfo monoacetone pentose.

4.973 mg. substance: 9.569 mg. CO_2 and 2.625 mg. H_2O
0.1171 gm.   "   : 0.0798 gm. $BaSO_4$
$C_{15}H_{20}O_7S$ Calculated.   C 52.32, H 5.88, S 9.31
344.2   Found.   " 52.48, " 5.91, " 9.36

The substance is very soluble in acetone and chloroform, fairly soluble in methyl and ethyl alcohol and ethyl acetate, somewhat soluble in hot water, boiling ether, and boiling heptane, slightly

soluble in boiling hexane, and almost insoluble in pentane either hot or cold.

Diacetyl Monoacetone Xylose—10 gm. of pure distilled monoacetone xylose were dissolved in 75 cc. of dry pyridine and 10.9 cc. (2.2 mols) of acetic anhydride were added. After 18 hours at room temperature, 2 cc. of water were added, and after an additional half hour at room temperature, the product was isolated as described for the compounds above. The washed, dried chloroform extract was concentrated under reduced pressure to a thick syrup and this was distilled. The boiling point was 121–126° (about 0.1 mm.). The syrup failed to crystallize. Its rotation, in chloroform, was $[\alpha]_D^{20} = \dfrac{-0.25° \times 100}{2 \times 2.024} = -6.1°$. The composition agreed with that of a diacetyl monoacetone pentose.

> 4.167 mg. substance: 8.110 mg. CO_2 and 2.465 mg. H_2O
> $C_{12}H_{18}O_7$.　Calculated.　C 52.53, H 6.62
> 274.1　　　Found.　　" 53.08, " 6.63

3-Acetyl-5-Benzoyl Monoacetone Xylose —15 gm. of crystalline 5-benzoyl monoacetone xylose were dissolved in 60 cc. of dry pyridine, cooled to about 10°, and 5.3 cc. (1.1 mols) of acetic anhydride were added. After standing overnight at room temperature, 2 cc. of water were added, and after an additional half hour the mixture was diluted with water and extracted with chloroform as described for the substances above. The washed, dried chloroform extracts were concentrated under reduced pressure to a syrup which was dissolved in ether, and cooled. Pentane was added just to turbidity, and after standing in the ice box the product crystallized. The mother liquors were reworked to give two additional crops, the total yield being 15.7 gm. or 91 per cent of the theoretical. The material was recrystallized twice from hexane giving a yield of 14.1 gm. (82 per cent) of pure product. It melted at 84.5–85.5° and had a rotation, in chloroform, of $[\alpha]_D^{22} = \dfrac{-1.06° \times 100}{2 \times 2.0} = -26.5°$. The composition corresponded to that of an acetyl benzoyl monoacetone pentose.

5.196 mg. substance: 11.564 mg. CO_2 and 2.880 mg. H_2O

$C_{17}H_{20}O_7$.   Calculated.   C 60.70, H 6.00

336.1   Found.   " 60.70, " 6.21

The product is very soluble in acetone, ethyl acetate, ether, and chloroform, somewhat soluble in methyl or ethyl alcohol, quite soluble in hot hexane or heptane, a little soluble in hot pentane, and almost insoluble in water, even hot.

5-Acetyl-3-p-Tolylsulfo Monoacetone Xylose—5 gm. of 5-acetyl monoacetone xylose were dissolved in 50 cc. of dry pyridine and 4.5 gm. (1.1 mols) of *p*-toluenesulfonchloride were added.   The mixture was shaken until the chloride dissolved and was then kept at 40° overnight.   After adding 1 cc. of water and standing for an additional half hour, the mixture was diluted with water, the product was extracted with chloroform, and worked up just as for the acetyl monoacetone.   The final syrupy product was taken up in ether from which it crystallized spontaneously in a few hours.   The crystals were filtered off and further crops were secured by working up the mother liquors.   The total yield was 4.2 gm. or 51 per cent of the theoretical.   The product was recrystallized from considerable hexane containing a little ether. After two recrystallizations the melting point was 84.5-85° and the rotation, in chloroform, was $[\alpha]_D^{20} = \dfrac{-1.32° \times 100}{2 \times 2.0} = -33.0°$.

The composition agreed with that of an acetyl tolylsulfo monoacetone pentose.

3.922 mg. substance: 7.595 mg. CO_2 and 2.100 mg. H_2O

0.1158 gm.   "   : 0.0698 gm. $BaSO_4$

$C_{17}H_{22}O_8S$.   Calculated.   C 52.82, H 5.74, S 8.30

386.2   Found.   " 52.81, " 6.01, " 8.28

The substance is very soluble in acetone, ether, ethyl acetate, and chloroform and quite soluble in methyl or ethyl alcohol.   It is moderately soluble hot, in water, heptane, and hexane but almost insoluble cold.   It is insoluble in pentane either hot or cold.

5-Benzoyl-3-p-Tolylsulfo Monoacetate Xylose—5 gm. of pure 5-benzoyl monoacetone xylose were dissolved in 30 cc. of dry pyridine, 3.6 gm. (1.1 mols) of *p*-toluenesulfonchloride were added, and the mixture was shaken till the chloride dissolved.   It was

then kept at 40° overnight.   1 cc. of water was added, and after
½ hour at room temperature, the mixture was diluted with water,
the product was extracted with chloroform, and worked up as for
the substances above.   The syrupy product crystallized immed-
iately upon adding ether.   The crystals were filtered off and the
mother liquors were reworked for a further quantity of substance.
The yield was 5.2 gm. or 68 per cent of the theoretical.   The
product was twice recrystallized from methyl alcohol.   It then
melted at 94.5–95.5° and had a rotation, in chloroform, of $[\alpha]_D^{20} =$
$\dfrac{-2.56° \times 100}{2 \times 2.0} = -64.0°$.   The composition agreed with that of a
benzoyl tolylsulfo monoacetone pentose.

4.621 mg. substance: 9.894 mg. CO_2 and 2.255 mg. H_2O
0.1133 gm.   "   : 0.0606 gm. $BaSO_4$
        $C_{22}H_{24}O_8S$. Calculated. C 58.92, H 5.40, S 7.15
        448.1   Found.   " 58.39, " 5.47, " 7.35

The substance is very soluble in acetone, ethyl acetate, and
chloroform, quite soluble in ether and in hot methyl or ethyl
alcohol, somewhat soluble in hot heptane and hexane, and only
very slightly soluble in water or pentane, either hot or cold.

In another preparation, 10 gm. of once crystallized 5-benzoyl
monoacetone xylose were treated as above.   The yield of crystal-
line material (m.p. 95–96°) was 8.4 gm.   The residue was re-
dissolved in pyridine and retolylsulfonated, giving an additional
0.8 gm. of pure product.   The total of 9.2 gm. is 60 per cent of
the theoretical.   The mother liquors from this second crop were
concentrated to a syrup and dried under reduced pressure to a
constant weight.   Yield, 3.6 gm.   An analysis showed only 1.4
per cent sulfur (theoretical 7.1 per cent) so that despite two tolyl-
sulfonations an appreciable fraction remains unsulfonated.

3-Acetyl-5-p-Tolylsulfo Monoacetone Xylose—5 gm. of pure
5-*p*-tolylsulfo monoacetone xylose were dissolved in 30 cc. of dry
pyridine and 1.5 cc. of acetic anhydride (1.1 mols) were added.
After standing overnight at room temperature, 1 cc. of water was
added, and after ½ hour more at room temperature the product
was extracted with chloroform and worked up as for the sub-
stances above.   The final syrupy material did not crystallize
nor did it do so after seeding with the crystalline 5-acetyl-3-*p*-

tolylsulfo monoacetone xylose. It was therefore dried under reduced pressure over phosphorus pentoxide and potassium hydroxide. Its rotation, in chloroform, was $[\alpha]_D^{20} = \dfrac{-0.77° \times 100}{2 \times 2.15}$ $= -17.9°$. The composition agreed only fairly well with that of an acetyl tolylsulfo monoacetone pentose.

3.383 mg. substance: 6.435 mg. CO_2 and 1.680 mg. H_2O
0.1328 gm.   "   : 0.0766 gm. $BaSO_4$
   $C_{17}H_{22}O_8S$. Calculated. C 52.82, H 5.74, S 8.30
   386.2   Found. " 51.88, " 5.56, " 7.92

3-Benzoyl-5-p-Tolylsulfo Monoacetone Xylose—5 gm. of 5-tolyl-sulfo monoacetone xylose were dissolved in 30 cc. of dry pyridine and 1.85 cc. (1.1 mols) of benzoyl chloride were added. After standing overnight at room temperature, 1 cc. of water was added, and after ½ hour at room temperature the mixture was diluted with water, the product was extracted with chloroform, and worked up as for the substances above. The washed, dried, chloroform extract was concentrated under reduced pressure to a syrup. This was dissolved in ether and the product immediately crystallized. The crystals were filtered off and the mother liquor was reworked to obtain further material. The yield was 5.7 gm. or 88 per cent of the theoretical. The substance was twice crystallized from methyl alcohol and then melted at 86–87°. Its rotation, in chloroform, was $[\alpha]_D^{20} = \dfrac{-1.48° \times 100}{2 \times 2.0} = -37.0°$.

The composition agreed with that of a benzoyl tolylsulfo mono-acetone pentose.

4.476 mg. substance: 9.689 mg. CO_2 and 2.150 mg. H_2O
0.1174 gm.   "   : 0.0626 gm. $BaSO_4$
   $C_{22}H_{24}O_8S$. Calculated. C 58.92, H 5.40, S 7.15
   448.1   Found. " 59.04, " 5.37, " 7.32

The substance is very soluble in acetone, ethyl acetate, and chloroform, fairly soluble in methyl and ethyl alcohol and in ether, only a little soluble in hot heptane and hexane, and almost insoluble in water and pentane.

5-Iodo Monoacetone Xylose—10 gm. of pure *p*-tolylsulfo mono-acetone xylose were dissolved in 100 cc. of acetone containing 10 gm. of sodium iodide, sealed in a flask, and heated in the steam

bath for 6 hours. The flask was cooled and opened, and the sodium toluene sulfonate was filtered off. The filtrate was concentrated under reduced pressure, water being added from time to time to remove all the acetone. The solution was finally concentrated to small volume, extracted several times with a relatively large volume of chloroform, and the extracts were washed with a little water. They were then dried with sodium sulfate, filtered with charcoal, and concentrated under reduced pressure to a syrup. This was taken up in ether, and on adding pentane, the product crystallized. The crystals were filtered off and further crops were obtained by working up the mother liquors. The yield of crude crystalline product was 7.9 gm. or 69 per cent of the theoretical. It was recrystallized by dissolving in 30 cc. of ethyl acetate and adding 200 cc. of hexane. On standing in the ice box the product crystallized in long fine needles. Recrystallized once more from hexane containing 5 per cent of ethyl acetate, it melted at 108–109° and had a rotation, in chloroform, of $[\alpha]_D^{22} =$

$$\frac{-1.60° \times 100}{2 \times 2.0} = -40.0°.$$ The composition agreed with that of

an iodo monoacetone pentose.

4.960 mg. substance: 5.925 mg. CO_2 and 2.090 mg. H_2O
4.305 "   "   : 3.362 " AgI
        $C_8H_{10}O_4I$. Calculated.   C 32.00, H 4 37, I 42.30
        300.0   Found   " 32.58, " 4.72, " 42.21

The substance is quite soluble in all the common organic solvents except hexane and heptane in which it is soluble hot and insoluble cold, and pentane in which it is only slightly soluble even when hot. It is fairly soluble in warm water but only slightly soluble in cold.

Monoacetone Xylose 5-Nitrate—20 gm. of pure 5-iodo monoacetone xylose and 20 gm. of silver nitrate were dissolved in 75 cc. of acetonitrile and refluxed for 2 hours over a free flame. 150 cc. of chloroform were added, the silver iodide was filtered off and extracted with hot chloroform, and the extract was washed three times with water. After drying with sodium sulfate it was concentrated under reduced pressure to a syrup, and this was taken up in warm ether. After cooling, pentane was added almost to turbidity and on seeding (the crystals were obtained by spontaneous evaporation of a small sample in air) the product crystal-

lized. It was filtered off and washed with pentane-ether and then pentane. Air-dried, the yield was 6.8 gm. or 43 per cent of the theoretical. It was twice recrystallized from ether and then had a melting point of 115.5–116.5°. The rotation, in chloroform, was

$[\alpha]_D^{20} = \dfrac{-0.99° \times 100}{2 \times 2.0} = -24.8°$. The composition agreed with

that of a pentose monoacetone nitrate.

5.110 mg. substance: 7.665 mg. CO_2 and 2.525 mg. H_2O
5.815   "   "   : 0.317 cc. N (at 26° and 753 mm.)
        $C_8H_{13}O_7N$. Calculated.   C 40 84, H 5.57, N 5.96
        235.1   Found.   " 40 91, " 5.54, " 6.17

The substance is quite soluble in methyl or ethyl alcohol, acetone, and ethyl acetate; fairly soluble, hot, in ether, chloroform, heptane, benzene, and water; somewhat soluble in cold ether and chloroform and in hot hexane; only slightly soluble, cold, in heptane, hexane, benzene, and water; almost insoluble in pentane even at the boiling point.

5-Benzylcarboxy Monoacetone Xylose—10 gm. of distilled crystalline monoacetone xylose were dissolved in 50 cc. of pyridine. The solution was cooled in running water and mechanically stirred and a solution of 9 gm. of benzyl carbonyl chloride in 20 cc. of chloroform was dropped in slowly, the addition requiring about 10 minutes. The mixture was allowed to stand overnight at room temperature, 2 cc. of water were added, and after another half hour at room temperature, water was added and the product was extracted with chloroform and worked up as for the substances above. The washed, dried chloroform extract was concentrated under reduced pressure to a syrup which was taken up in boiling ether. After a short time in the ice box the product crystallized spontaneously. The yield, in two crops, was 7.2 gm. or 42 per cent of the theoretical. The product was twice recrystallized from ether. It then melted at 90–91° and had a rotation, in

chloroform, of $[\alpha]_D^{20} = \dfrac{+0.30° \times 100}{2 \times 2.0} = +7.5°$. Its composition

agreed with that of a benzylcarboxy monoacetone pentose.

4.435 mg. substance: 9.690 mg. CO_2 and 2.575 mg. H_2O
        $C_{16}H_{20}O_7$. Calculated.   C 59.22, H 6.22
        324.2   Found.   " 59.59, " 6.50

The substance is very soluble at room temperature in all the common organic solvents with the exception of heptane, hexane, and pentane. It is fairly soluble in hot heptane and hexane but is almost insoluble cold. In pentane it is nearly insoluble either hot or cold. The substance is nearly insoluble in cold water but a little soluble hot and crystallizes well on cooling the solution.

To study the hydrolysis of this substance by acid, a weighed sample was shaken with 0.05 N hydrochloric acid but was found to be only slightly dissolved in 20 hours. Another sample was dissolved in dioxane and an equal volume of 0.1 N hydrochloric acid was added. No appreciable change in rotation occurred in 15 hours. Another portion, dissolved in dioxane, was added to an equal volume of 0.1 N sodium hydroxide and portions were

TABLE I

Reaction of Substituted Tolylsulfo Monoacetone Xylose with Sodium Iodide in Acetone

Assigned structure	Per cent of tolylsulfo removed when heated at 100° for	
	2 hrs	6 hrs
5–Tolylsulfo....	67	88
3–Acetyl-5-tolylsulfo	32	59
5–Acetyl-3-tolylsulfo	6	4
3–Benzoyl-5-tolylsulfo.	38	62
5–Benzoyl-3-tolylsulfo	3	5

titrated at intervals with acid. It was found that 1 equivalent of alkali reacted almost instantly at room temperature and a second equivalent within 15 minutes.

Reaction of Tolylsulfo Xyloses with Sodium Iodide in Acetone— Inasmuch as Oldham and Rutherford[2] have shown that the tolyl-sulfo group on a primary alcoholic group reacts with sodium iodide in acetone more rapidly than if on a secondary alcoholic group, this property was used to confirm the structures assigned to the tolylsulfo derivatives. 0.100 gm. of substance was dissolved in a standardized solution of sodium iodide in acetone and diluted with acetone to exactly 5.0 cc. Portions of this solution were sealed in Pyrex test-tubes and heated in the steam bath for 2 and for 6 hours. The tubes were then removed, cooled, and opened.

Measured volumes were titrated for iodide with silver nitrate-ammonium thiocyanate in the usual manner, with ferric alum as indicator. Preliminary experiments had shown that glucose derivatives of known structure gave, within fair limits of accuracy,
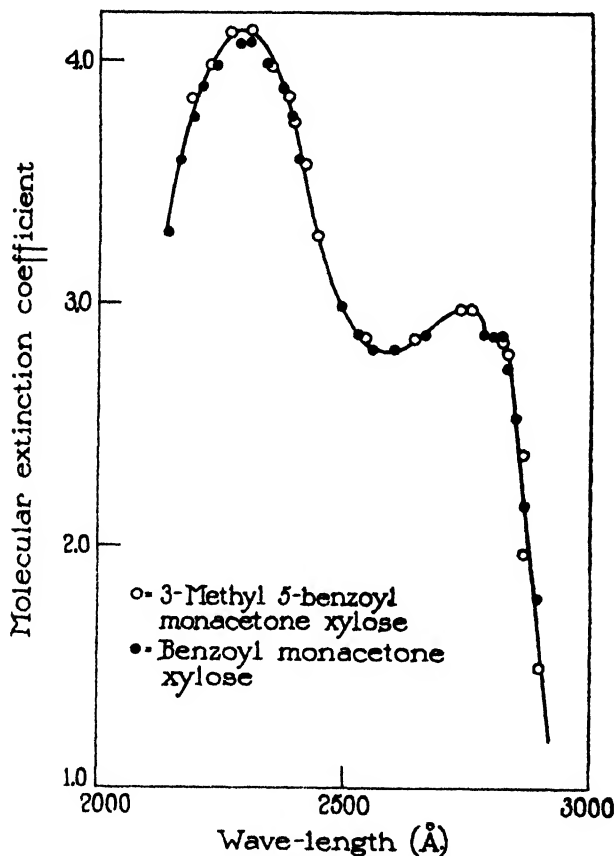
Fig. 1. Molecular extinction coefficient of 3-methyl-5-benzoyl monoacetone xylose and benzoyl monoacetone xylose.

the results which would have been expected on the assumption that none of the organic iodide reacts with silver nitrate under the conditions used. The results with the xylose derivatives are given in Table I, the actual data being omitted for the sake of brevity.

Ultra-Violet Absorption of 5-Benzoyl Monoacetone Xylose[4]—
3-Methyl-5-benzoyl monoacetone xylose (the preparation of this
substance will be given later) and the presumed 5-benzoyl mono-
acetone xylose were dissolved in alcohol and their molecular
extinction coefficients $(I_0/I = 10^{\epsilon c})$ were determined in the
ultra-violet region with the sector method. The region corre-
sponding to the benzoyl group is reproduced in Fig. 1 from which
it will be seen that the two compounds have almost identical
absorptions. On this basis the orthobenzoate structure may be
excluded for the monobenzoyl monoacetone xylose.

[4] This section was prepared by Dr. A. Rothen of this department.

3-METHYL XYLOSE AND 5-METHYL XYLOSE

By P. A. LEVENE and ALBERT L. RAYMOND

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In a previous publication[1] there was described the preparation of a monobenzoyl monoacetone xylose, with the acyl group presumably in position (5). It was remarked at that time that there was reason to question the homogeneity of the product although experiments then cited seemed to indicate it to be a single substance. The present paper deals with the observations which were the basis of our doubts.

The crystalline benzoyl monoacetone xylose was methylated by the Purdie method in order to secure the 3-methyl derivative, and there was obtained a crystalline methyl benzoyl monoacetone xylose. This was debenzoylated to a syrupy methyl monoacetone derivative which on acid hydrolysis gave a crystalline methyl xylose. The substance has the methyl group in position (3) and not in (5), for on treatment with methyl alcoholic hydrogen chloride the optical rotation indicated the formation of both a normal and a γ-glycoside. This 3-methyl xylose was also prepared from trityl monoacetone xylose by methylation, followed by removal of the trityl and acetone groups.

In the initial methylation of the benzoyl monoacetone xylose, however, the yield of the crystalline 5-benzoyl-3-methyl monoacetone xylose was only 40 per cent of the theoretical and it was found that much of the remainder was accounted for by the formation of an isomeric, syrupy methyl benzoyl monoacetone pentose. On debenzoylation of this syrup a beautifully crystalline methyl monoacetone pentose was obtained and this, on acid hydrolysis, gave a syrupy methyl pentose, shown by the glycoside formation to be the 5-methyl derivative. This substance was evidently the result of a complex reaction if the starting material was really 5-benzoyl monoacetone xylose, and because of this the configuration of the resulting 5-methyl pentose became uncertain.

[1] Levene, P. A., and Raymond, A. L., *J. Biol. Chem.*, **102**, 317 (1933).

Fortunately, a more convenient method was found for its preparation. On treating the 5-tolylsulfo monoacetone xylose previously described[1] with methyl alcohol and sodium methylate at room temperature, it was converted in quite good yield to a crystalline anhydro monoacetone derivative. This, in turn, heated with methyl alcoholic sodium methylate, opened the ring and gave a crystalline methyl monoacetone pentose identical with that described above. In order to determine its configuration the methyl monoacetone pentose was further methylated to the dimethyl derivative, this was hydrolyzed to the free dimethyl pentose, oxidized to the acid, converted to the lactone, and finally to the crystalline phenylhydrazide. The derivatives, at every stage, had the properties given by Haworth and Porter[2] for the corresponding dimethyl xylose derivatives, but for complete assurance monoacetone xylose was methylated and carried through the same series of reactions. The derivatives agreed at each step, and a mixed melting point on the phenylhydrazides showed no depression. It was therefore certain that ribose was not produced by opening the oxidic ring.

This fact is of interest in another connection, for Robinson[3] has suggested the formation of ribose, due to Walden inversion, on hydrolyzing 3-phospho xylose. It would seem that in the case of the anhydro sugar where opening of the ring must involve detachment of the oxygen from a carbon atom, greatest opportunity for inversion would occur. That it does not occur would appear significant.

It may be mentioned that the 5-methyl xylose reduces Fehling's solution even at room temperature in a few minutes. Being a true γ sugar it should afford opportunity to investigate other properties of the γ sugars and this we intend to do.

While the evidence is not yet complete, it would appear that the assumption of a migration best explains the formation of 3-methyl-5-benzoyl and 5-methyl-3-benzoyl monoacetone xylose upon methylation of what seems to be a single benzoyl monoacetone xylose.

EXPERIMENTAL

3-Methyl-5-Benzoyl Monoacetone Xylose—80 gm. of crystalline 5-benzoyl monoacetone xylose were methylated by the Purdie

[2] Haworth, W. N., and Porter, C. R., *J. Chem. Soc.*, 611 (1928).
[3] Robinson, R., *Nature*, **120**, 44 (1927).

method, with 400 cc. of methyl iodide, and 300 gm. of silver oxide in six portions. The product was isolated into chloroform in the usual manner and the chloroform solution was concentrated under reduced pressure to a syrup. This was taken up in methyl alcohol, the solution was cooled, and water was added almost to turbidity. On cooling and seeding (the seed crystals were obtained by distilling a portion of the product under greatly reduced pressure) the product crystallized. The yield, however, was only 30 gm. The syrupy residue was dissolved in chloroform, dried and remethylated as before but with only 0.3 the reagents first employed. Treated as above this material deposited an additional 5 gm. of crystalline product. The syrupy residue was again taken up in chloroform, washed and dried, and distilled under reduced pressure (about 0.1 mm.). The material contained some (4 gm.) low-boiling material (110–125°) but the rest came over at a constant temperature (164–167°). Yield 36 gm. $[\alpha]_D^{20} = \dfrac{-1.38° \times 100}{2 \times 2.092} = -33.0°$ (in chloroform). The composition agreed with that of a methyl benzoyl monoacetone pentose.

5.633 mg. substance: 12.915 mg. CO_2 and 3.205 mg. H_2O
4.522 " " : 3.620 " AgI
$\quad$ $C_{16}H_{20}O_6$ Calculated. C 62.30, H 6.54, OCH_3 10.06
$\quad$ 308.2 $\quad$ Found. " 62.55, " 6.37, " 10.57

The crystalline 3-methyl-5-benzoyl monoacetone xylose was recrystallized from hot heptane, from which it crystallized in beautiful large prisms on cooling to room temperature. After one more recrystallization the product melted at 64–65.5° and had a rotation, in chloroform, of $[\alpha]_D^{20} = \dfrac{-2.01° \times 100}{2 \times 2.0} = -50.2°$. A portion of the material was melted and rapidly cooled. Its refractive index was $n_D^{20} = 1.5052$. The composition agreed with that of a methyl benzoyl monoacetone pentose.

4.250 mg. substance: 9.705 mg. CO_2 and 2.595 mg. H_2O
8.390 " " : 6.340 " AgI
$\quad$ $C_{16}H_{20}O_6$. Calculated. C 62.30, H 6.54, OCH_3 10.06
$\quad$ 308.2 $\quad$ Found. " 62.28, " 6.84, " 9.98

The substance is almost insoluble in water and in pentane even at the boiling points. In boiling hexane and heptane it is quite soluble and crystallizes beautifully on cooling. In the other

common organic solvents it is quite soluble at room temperature.

The 36 gm. of syrupy methyl benzoyl monoacetone xylose were dissolved in 300 cc. of dry methyl alcohol and 2 cc. of a 1.7 N solution of barium oxide in methyl alcohol were added. After standing overnight at room temperature the mixture was distilled under reduced pressure, water being added to effect removal of the methyl benzoate. The aqueous solution was filtered with charcoal and concentrated under reduced pressure to a small volume. On standing overnight in the ice box, a considerable portion crystallized. The crystals were filtered from the residual syrup and two further crops were secured by adding pentane to the solution of the syrup in ether. The final non-crystalline material was concentrated and dried to constant weight. The syrup weighed 12.5 gm. and the crystals 8.3 gm. A rotation, melting point, and mixed melting point established the crystalline product as identical with the 5-methyl monoacetone xylose described below.

5-Benzoyl monoacetone xylose
80 gm.   0.272 mol

Crystals
3-Methyl-5-benzoyl mono-
acetone xylose
35 gm.   0.107 mol
39 per cent

Syrup
Dimethyl monoacetone
xylose
4 gm.   0.018 mol
7 per cent

Syrup
Methyl benzoyl mono-
acetone xylose
36 gm.   0.117 mol
43 per cent

Crystals
5-Methyl monoacetone
xylose
8.3 gm.   0.041 mol
15 per cent

Syrup
Methyl monoacetone
xylose
12.5 gm.   0.061 mol
22 per cent

Crystals
3-Methyl xylose
1.8 gm.   0.011 mol
4 per cent

Syrup
Methyl xylose
7.4 gm.   0.045 mol
15 per cent

The syrupy product was primarily 3-methyl monoacetone xylose, identical with that described above. It was hydrolyzed with acid and gave a further quantity of crystalline 3-methyl xylose. The weights of the various fractions and the percentage yields of each are given in the accompanying diagram. It is estimated that methylation of the supposedly pure 5-benzoyl monoacetone xylose gave about three-fourths 3-methyl and one-fourth 5-methyl derivative.

3-Methyl Monoacetone Xylose—20 gm. of the pure crystalline 3-methyl-5-benzoyl monoacetone xylose were dissolved in 150 cc. of dry methyl alcohol and 1 cc. of a 1.7 N solution of barium oxide in methyl alcohol was added. After standing overnight at room temperature the mixture was concentrated under reduced pressure, filtered with charcoal, concentrated to a syrup, and distilled under greatly reduced pressure. The yield of distilled syrup was 11.1 gm. or 84 per cent of the theoretical. It did not crystallize, nor did it on seeding with the beautifully crystalline 5-methyl monoacetone xylose. The rotation, in water, was $[\alpha]_D^{27} = \dfrac{-2.48° \times 100}{2 \times 2.288} = -54.1°$ and the refractive index was $n_D^{20} = 1.4589$. The composition agreed with that of a methyl monoacetone pentose.

3.959 mg. substance: 7.700 mg. CO_2 and 2.835 mg. H_2O
8.306 "   "   : 9.490 " AgI
        $C_9H_{16}O_5$. Calculated. C 52.91, H 7.90, OCH_3 15.19
        204.1   Found.   " 53.04, " 8.02,   "   15.09

3-Methyl Xylose—10 gm. of 3-methyl monoacetone xylose were dissolved in 100 cc. of 0.1 N sulfuric acid and heated at 80° for 2 hours. After cooling, the sulfuric acid was removed by shaking the solution with barium carbonate, and the clear, filtered solution was concentrated under reduced pressure to a syrup. This was placed in a crystallizing dish over phosphorus pentoxide and sodium hydroxide, and left overnight under greatly reduced pressure. In the morning it was found to have crystallized to a hard solid mass. The cake was dissolved in 100 cc. of ethyl acetate containing 4 per cent of methyl alcohol. The solution was filtered, and on cooling crystallized spontaneously. The product was recrystallized in the same manner. The yield of

pure substance was 6.3 gm. or 80 per cent of the theoretical. It melted at 103–104° and had a rotation, in water, of $[\alpha]_D^{20} =$

$$\frac{+2.09° \times 100}{2 \times 2.0} = +52.2°,$$ $\frac{3}{4}$ minute after dissolving, and $+14.8°$

at equilibrium after 4 hours. The composition agreed with that of a methyl pentose.

5.220 mg. substance: 8.415 mg. CO_2 and 3.510 mg. H_2O
4.912 "   "   : 7.101 "   AgI
　　$C_6H_{12}O_5$.   Calculated.   C 43.88, H 7.37, OCH_3 18.89
　　164.1   Found.   " 43.96, " 7.53,   "   19.09

The substance reduces Fehling's solution on gentle warming.

0.5 gm. of the crystalline sugar was mixed with 1.7 gm. (3 mols) of p-bromophenylhydrazine, dissolved in a little glacial acetic acid, diluted with water, and heated on the boiling water bath. The osazone formed fairly rapidly with but little accompanying tar. After 2 hours on the bath, it was cooled and allowed to stand in the ice box. The osazone was filtered off, dissolved in a little alcohol, and water was added to faint opalescence. On warming the mixture the osazone separated in crystalline form, and crystallization was completed in the cold. The product was recrystallized once more in the same manner and was then a felt of coarse yellow needles. On heating, it partially melted at 142–143° with considerable darkening and melted completely at 153–155°. Its rotation, in pyridine-absolute alcohol (2:3 by

volume) was $[\alpha]_D^{20} = \dfrac{+0.03° \times 100}{0.5 \times 1.0} = +6.0°,$ initial, and $-14.0°$

at equilibrium after 24 hours. The composition agreed with that of a bromophenylosazone of a methyl pentose.

4.435 mg. substance: 0.449 cc. N at 25° and 759 mm.
7.262 "   "   : 3.350 mg. AgI
　　$C_{18}H_{20}O_3N_4Br_2$.   Calculated.   N 11.21, OCH_3 6.20
　　500.0   Found.   " 11.59,   "   6.09

5-Trityl Monoacetone Xylose—19 gm. of crystalline monoacetone xylose were dissolved in 150 cc. of dry pyridine and 29.5 gm. of triphenylchloromethane were added. The mixture was shaken until all the chloride dissolved and was then placed in the hot room at 40° for 2 hours. It was next heated on the steam bath

for an additional $1\frac{1}{2}$ hours and was then concentrated under reduced pressure to remove most of the pyridine. Water and chloroform were added and three chloroform extracts of the aqueous layer were made. The combined extracts were washed with acid and treated as described for the substances above. The dry extracts were concentrated under reduced pressure to a syrup which was dissolved in hot heptane containing 1 per cent of ethyl acetate. On seeding and cooling (the seed crystals were secured by spontaneous evaporation of a portion of the material) the product crystallized. The yield, in two crops, was 24 gm. or 56 per cent of the theoretical. The material was twice recrystallized from heptane containing 3 per cent of ethyl acetate and it then melted at 118-119° and had a rotation, in chloroform, of $[\alpha]_D^{25} = \dfrac{+0.42° \times 100}{2 \times 2.0} = +10.5°$. The composition agreed with that of a triphenylmethyl monoacetone pentose.

4.081 mg. substance: 11.220 mg. CO_2 and 2.515 mg. H_2O

$C_{27}H_{28}O_5$.   Calculated.   C 74.97, H 6.53

432.2   Found.   " 74.98, " 6.90

The substance is very soluble in chloroform, acetone, and ethyl acetate, quite soluble in methyl or ethyl alcohol and ether, insoluble in water. In boiling heptane and hexane it is quite soluble and in boiling pentane a little soluble. It is very little soluble in these three at room temperature.

3-Methyl 5-Trityl Monoacetone Xylose—15 gm. of pure trityl monoacetone xylose were dissolved in 50 cc. of methyl iodide and a Purdie methylation was performed with 24 gm. of silver oxide in six portions. The product was isolated in the usual manner and remethylated exactly as above. A portion of the product was dried and analyzed. The methoxyl content was lower than the theoretical so the material was once more methylated and isolated as above. The chloroform solution was dried and concentrated under reduced pressure to a syrup. On standing, the syrup crystallized but it has not as yet been recrystallized from a solvent. The rotation, in chloroform, was $[\alpha]_D^{22} = \dfrac{-1.69° \times 100}{2 \times 2.068} = -40.9°$. The composition agreed only fairly well with that of a methyl triphenylmethyl monoacetone pentose.

3.955 mg. substance: 10.800 mg. CO_2 and 2.405 mg. H_2O
8.180 "   "   : 3.700 " AgI

$C_{28}H_{30}O_5$.   Calculated.   C 75.30, H 6.78, OCH_3 6.94
446.2   Found.   " 74.47, " 6.81,   "   5.97

3-Methyl Xylose from the Trityl Derivative—25 gm. of amorphous 3-methyl 5-trityl monoacetone xylose were dissolved in 60 cc. of glacial acetic acid and cooled in ice. 15 cc. of a cold saturated solution of hydrogen bromide in acetic acid were added, and after a minute in the ice bath, the mixture was poured into ice water and diluted to 300 cc. The trityl derivatives were filtered off and the mixture was heated at 80° for $1\frac{1}{4}$ hours. After cooling in ice, moist silver carbonate was added until the inorganic bromide was removed. The mixture was filtered and the silver precipitate was washed with dilute acetic acid. The filtrates were saturated with hydrogen sulfide, aerated, filtered, and concentrated under reduced pressure to a syrup. This was taken up in dry methyl alcohol containing a little barium methylate and allowed to stand $\frac{1}{2}$ hour at room temperature. The solution was then concentrated under reduced pressure to a syrup which was extracted several times with boiling ethyl acetate containing 1 per cent of methyl alcohol. The extracts were combined, concentrated on the steam bath, and allowed to cool. On seeding with 3-methyl xylose the product crystallized. It was filtered off, washed with ethyl acetate, and dried. The yield was 2.4 gm. or 25 per cent of the theoretical. The product was once recrystallized from ethyl acetate containing 3 per cent of methyl alcohol and it then had a rotation, in water, of $[\alpha]_D^{22} = \dfrac{+2.23° \times 100}{2 \times 2.004} = +55.6°$ after 3 minutes and $[\alpha]_D^{22} = \dfrac{+0.69° \times 100}{2 \times 2.004} = +17.2°$ at equilibrium. It melted at 102–103.5° and a mixed melting point with the 3-methyl xylose prepared previously was 101.5–103°.

1,2-Monoacetone-3,5-Anhydro Xylose—100 gm. of once crystallized 5-tolylsulfo monoacetone xylose were added to a cooled (15°) solution of 13.4 gm. (2 mols) of sodium in 700 cc. of dry methyl alcohol, and shaken until dissolved, about 1 hour. The solution was kept overnight at room temperature (20–25°) and then, without filtering, was concentrated under reduced pressure. Water was added to keep the sodium toluene sulfonate in solution

and the distillation was continued until the alcohol was almost entirely removed. The solution was extracted with 2 liters of chloroform in five portions and the combined extracts were washed with a little water and dried with sodium sulfate. The chloroform solution was concentrated under reduced pressure to a syrup which was distilled under reduced pressure. The boiling point was 63 65° (about 0.1 mm.) The product crystallized in the ice box. The yield was 45 gm. or 90 per cent of the theoretical. Its melting point was $16.9 - 17.3°$ and its rotation was $[\alpha]_D^{20} = \dfrac{+0.57° \times 100}{2 \times 2.44} = +11.7°$ in chloroform, and $[\alpha]_D^{20} = \dfrac{+0.60° \times 100}{2 \times 2.04} = +14.7°$ in water. $n_D^{20} = 1.4542$. The composition agreed with that of a monoacetone anhydro pentose.

3.800 mg. substance: 7.800 mg. CO_2 and 2 365 mg. H_2O
$C_8H_{12}O_4$. Calculated. C 55 78, H 7.03
172 1   Found.   " 55.98, " 6 97, OCH_3 none

The product is soluble in all the common organic solvents and in water. It may be crystallized from methyl alcohol by cooling to about 5°.

5-Methyl Monoacetone Xylose This product was prepared in three ways: First, by refluxing monoacetone anhydro xylose for 18 hours with a methyl alcohol solution containing 2 mols of sodium methylate (yield 56 per cent), second, by heating a similar mixture of monoacetone anhydro xylose and methyl alcoholic sodium methylate in sealed tubes for 8 hours in the boiling water bath, or third, most easily, by dissolving 5-tolylsulfo monoacetone in methyl alcohol with 3 mols of sodium methylate and heating in sealed tubes for 4 hours in the boiling water bath.

In the first case the reaction was followed polarimetrically and the curve is given in Fig. 1 as being of interest for the rate of opening of a propylene oxide ring.

For preparing the 5-methyl monoacetone xylose by the third procedure, 180 gm. of once crystallized 5-tolylsulfo monoacetone xylose were added to a cooled (15°) solution of 36 gm. (3 mols) of sodium in 500 cc. of methyl alcohol, and shaken until dissolved (about ½ hour). The mixture, from which sodium toluene sulfonate had already begun to separate, was sealed in Pyrex flask-

bodies and heated in the boiling water bath for 4 hours. The
bulbs were cooled and opened and water was added. The mixture
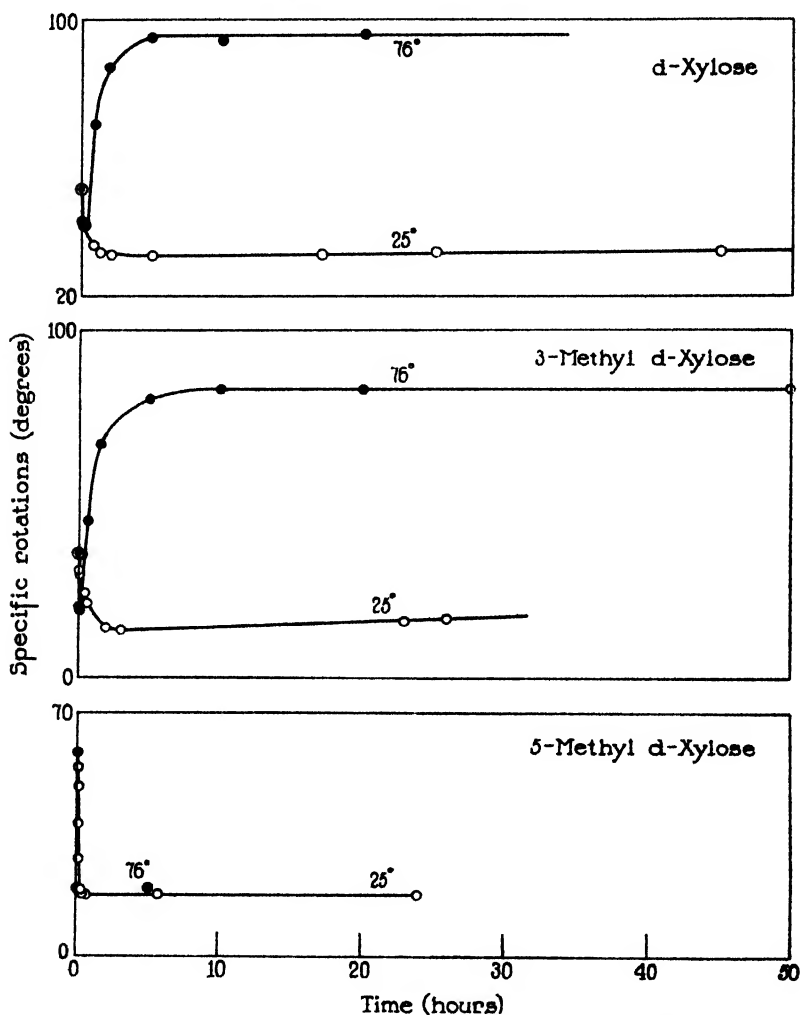
FIG. 1. Specific rotations during glycoside formation of xylose, 3-methyl
xylose, and 5-methyl xylose.

was concentrated under reduced pressure, with occasional addi-
tion of water, until the methyl alcohol was removed. The

aqueous solution was extracted with considerable chloroform in several portions and the combined extracts were washed with a little water and dried with sodium sulfate. The chloroform solution was concentrated under reduced pressure to a syrup which crystallized spontaneously in a few minutes. The material was dissolved in warm ether, cooled, and seeded. From the mother liquors of this crystallization on concentration, two additional crops were secured, the total yield being 84 gm. or 78 per cent of the theoretical. After two further crystallizations from ether the material melted at 80.5 − 81.5° and had a rotation, in water,

of $[\alpha]_D^{20} = \dfrac{-0.81° \times 100}{2 \times 2.0} = -20.2°$.

For a substance apparently identical with the above, Svanberg[4] found a melting point of 78° and $[\alpha]_{Hg\ yellow}^{18} = -21.4°$ (in water).

The composition agreed with that of a methyl monoacetone pentose.

4.980 mg. substance: 9.635 mg. CO_2 and 3.585 mg. H_2O
7.102 "   "   : 8.424 " AgI
$C_9H_{16}O_5$. Calculated. C 52.91, H 7.90, OCH_3 15.19
204.1   Found.   " 52.77, " 8.06, " 15.66

The product is soluble in water and in all the common organic solvents except pentane, hexane, and heptane in which it is almost insoluble in the cold. At the boiling point, however, it is fairly soluble in heptane and hexane, and slightly soluble in pentane. It crystallizes well from fairly strong ethereal solution on cooling in the ice box, or from hexane on cooling to room temperature.

5-Methyl Xylose—10 gm. of the pure crystalline 5-methyl monoacetone were dissolved in 100 cc. of 0.1 N sulfuric acid by warming and heated at 80° for 2 hours. The solution was cooled and the acid was removed by shaking with moist barium carbonate. The filtrate was concentrated under reduced pressure, filtered with charcoal, and concentrated to a syrup which was dried over potassium hydroxide and phosphorus pentoxide under greatly reduced pressure. All attempts at crystallization have been thus far unsuccessful.

Although a syrup, the product showed a slight mutarotation

[4] Svanberg, O., *Ber. chem. Ges.*, **56**, 2195 (1923).

in water, the value after 3 minutes being $[\alpha]_D^{20} = \dfrac{+1.35° \times 100}{2 \times 2.056}$

$+32.8°$, and at equilibrium, after 4 hours, $+36.4°$.

For a substance apparently identical with the above, Svanberg[4] found $[\alpha]_{Hg \; yellow}^{18} = +40.5°$ to $+42.0°$ (in water).

The composition agreed with that of a methyl pentose.

5.391 mg. substance: 8.620 mg. CO_2 and 3.465 mg. H_2O
6.895 "   "   : 9.900 " AgI
$\quad$ $C_6H_{12}O_5$. Calculated. C 43.88, H 7.37, OCH_3 18.89
$\quad$ 164.1   Found.   " 43.61, " 7.20, "   18.96

The substance reduced Fehling's solution after a few minutes at room temperature or immediately on gentle warming.

It was converted to the p-bromophenylosazone in exactly the manner described for the 3-methyl xylose.   The osazone formed a little more rapidly than in the case of the 3-methyl osazone and was accompanied by more tar formation.   The osazone was less soluble than in the case of that from the 3-methyl.   It was twice crystallized from alcohol and was then a mat of flat, yellow needles. It melted, with darkening, at 170–171°.   Its rotation, in pyridine-absolute alcohol (2:3 by volume) was $[\alpha]_D^{20} = \dfrac{-0.25° \times 100}{0.5 \times 1.0} =$

$-50.0°$, initial, and $-30.0°$ at equilibrium, after 24 hours.   Its composition agreed with that of a bromophenylosazone of a methyl pentose.

5.150 mg. substance: 0.519 cc. N (at 25° and 758 mm.)
5.710 "   "   : 2.595 mg. AgI
$\quad$ $C_{18}H_{20}O_3N_4Br_2$. Calculated. N 11.21, OCH_3 6.20
$\quad$ 500 0   Found   " 11.52, "   6.00

Methylation and Oxidation of Monoacetone Xylose—21 gm. of pure monoacetone xylose were methylated by the Purdie method, with 200 cc. of methyl iodide and 180 gm. of silver oxide in six portions.   The product was isolated in the usual way and re-methylated with 140 cc. of methyl iodide and 120 gm. of silver oxide in six portions.   The product was isolated and distilled. The yield was 19.4 gm.   The rotation, in water, was $[\alpha]_D^{20} = \dfrac{-1.85° \times 100}{2 \times 2.02} = -45.8°$. $n_D^{20} = 1.4424$.   Haworth and Porter[2]

found $[\alpha]_{5780}^{15} = -46.6°$ and $n_D^{15} = 1.4455$. The methoxyl content was 29.5 per cent (calculated 28.4 per cent for the dimethyl monoacetone xylose).

6.1 gm. of the above material were dissolved in 45 cc. of warm 0.1 N hydrochloric acid and heated at 80° for 2 hours. The solution was cooled and distilled under reduced pressure to remove the liberated acetone and then oxidized with barium hypoiodite in the usual manner. The product was isolated and distilled under reduced pressure. The yield was 2.8 gm. The methoxyl content was 35.1 per cent (calculated 35.6 per cent). The rotation, in water, was $[\alpha]_D^{22} = \dfrac{+3.40° \times 100}{2 \times 2.088} = +81.4°$ (initial). $n_D^{20} = 1.4621$.

Haworth and Porter gave for the dimethyl xylonic lactone $[\alpha]_{5780}^{21.5} = +81.5°$ and $n_D^{15} = 1.4643$.

Methylation and Oxidation of 5-Methyl Monoacetone Xylose— 15 gm. of pure 5-methyl monoacetone xylose were methylated exactly as described for the monoacetone xylose and the product was isolated and remethylated. After distillation there was obtained 13.5 gm. of product with $[\alpha]_D^{22} = \dfrac{-1.98° \times 100}{2 \times 2.108} = -46.9°$ (H_2O). $n_D^{20} = 1.4432$. The methoxyl content was 28.1 per cent (calculated 28.4 per cent).

5 gm. of this material were hydrolyzed and oxidized in the same manner as for the corresponding product from the monoacetone xylose. The yield of distilled dimethyl lactone was 2.4 gm. The rotation, in water, was $[\alpha]_D^{22} = \dfrac{+3.44° \times 100}{2 \times 2.088} = +82.4°$ (initial). $n_D^{20} = 1.4620$. The methoxyl content was 35.4 per cent (calculated 35.6 per cent).

In a repetition of this procedure, 3.4 gm. of distilled lactone were obtained from 5.1 gm. of dimethyl monoacetone xylose.

Identity of Phenylhydrazides of Dimethyl Xylonic Lactones— 2.6 gm. of the lactone from the methylated monoacetone xylose, and 3.1 gm. of the lactone from the methylated 5-methyl monoacetone xylose were mixed with equimolal quantities of phenylhydrazine in ethereal solution and converted to the phenylhydrazides exactly as described by Haworth and Porter. The trituration

with ether was omitted and the products were crystallized from benzene containing a little absolute alcohol. They were recrystallized by dissolving in a little warm ethyl acetate, adding several volumes of benzene, and stirring. The products both had the composition of phenylhydrazides of dimethyl pentonic lactones.

From Methylation of Monoacetone Xylose—Yield of phenylhydrazide, 2.8 gm. (67 per cent of the theoretical). $[\alpha]_D^{20} = \dfrac{+0.24° \times 100}{2 \times 2.00} = +6.0°$ (absolute alcohol). The rotation was not reported by Haworth and Porter. Melting point, 95–96° (Haworth and Porter 94–95°). The composition of the substance agreed with that of the phenylhydrazide of a dimethyl pentonic acid lactone.

4.865 mg. substance: 9.770 mg. CO_2 and 3.095 mg. H_2O
5.581 "   "   : 0.499 cc. N (at 26° and 752 mm.)
        $C_{13}H_{20}O_5N_2$.   Calculated.   C 54.89, H 7.09, N 9.86
        284.2   Found.   " 54.77, " 7.13, " 10.10

From Methylation of 5-Methyl Monoacetone Xylose—Yield of phenylhydrazide, 3.4 gm. (68 per cent of the theoretical). $[\alpha]_D^{20} = \dfrac{+0.34° \times 100}{2 \times 2.00} = +8.5°$ (absolute alcohol). Melting point, 95–96°. Mixed melting point with sample above 95–96°. The composition of the substance agreed with that of the phenylhydrazide of a dimethyl pentonic acid lactone.

4.720 mg. substance: 9.510 mg. CO_2 and 3.100 mg.H_2O
5.285 "   "   : 0.460 cc. N (at 24° and 754 mm.)
        $C_{13}H_{20}O_5N_2$.   Calculated.   C 54.89, H 7.09, N 9.86
        284.2   Found.   " 54.95, " 7.36, " 9.93

Osazone of 3,5-Dimethyl Xylose—5.6 gm. of 3,5-dimethyl monoacetone xylose were dissolved, with shaking, in 56 cc. of warm 0.2 N sulfuric acid and heated at 80° for 2 hours. The solution was cooled and shaken with moist barium carbonate until neutral and filtered. An aliquot portion, equivalent to 0.5 gm. of dimethyl xylose, was mixed with acetic acid and a calculated 3 mols of p-bromophenylhydrazine, and heated on the steam bath. An oil separated and the aqueous layer was decanted. The oil was taken up in warm methyl alcohol, from which it

crystallized on cooling. The osazone was filtered off, dissolved in warm methyl alcohol, and water was added to opalescence. The osazone crystallized immediately on cooling. It was filtered off, washed on the filter with a very little methyl alcohol added dropwise, and dried. It melted at 107–108.5° without decomposition and had a rotation in pyridine-absolute alcohol (2:3 by

TABLE I

Observed Rotations during Glycoside Formation at 25° and 76° of 3-Methyl and 5-Methyl Xylose

Time		25° (measured in 4 dm. tube)		76° (measured in 2 dm. tube)	
		3-Methyl	5-Methyl	3-Methyl	5-Methyl
hrs.	*min.*				
	1½	0.65	1.32		
	3	0.67	1.23		
	5	0.70	1.01	0.23	0 22
	7	0.70	0 86		
	10	0.71	0.63	0 22	
	18	0.66	0 43		
	20			0 40	
	25	0 55	0 40		
	40	0 49	0 40	0 51	
1		0.43			
1	30			0.76	
2		0.33			
3			0.40	0.88	
4		0.31			
5			0.41	0.91	0.22
10				0.94	
20				0.94	
24		0.37	0.40		
50		0.39		0.93	

volume) of $[\alpha]_D^{22} = \dfrac{-0.23° \times 100}{0.5 \times 1.0} = -46°$, 3 minutes after dissolving, and $-30°$ at equilibrium. The composition agreed fairly well with that of a bromophenylosazone of a dimethyl pentose.

5.210 mg. substance: 0.504 cc. N (at 33.5° and 751 mm.)
5.890 "   "   : 5.015 mg. AgI

$C_{19}H_{22}O_4N_4Br_2$. Calculated. N 10.91, OCH_3 12.06
514.1   Found.   " 10.65, "   11.24

Glycoside Formation of 3-Methyl and 5-Methyl Xylose—The procedure employed was that previously described.[5]   0.0344 M[6] solutions of the sugars in methyl alcohol containing 0.5 per cent of hydrogen chloride were used, and two temperatures, room temperature (about 25°) and 76°, were employed.   At the higher temperature the samples were heated in sealed tubes in a bath of boiling carbon tetrachloride for the desired lengths of time and the rotations were read in 2 dm. tubes at 25°.   The room temperature experiments were read in 4 dm. tubes.   The observed rotations are given in Table I and the calculated specific rotations are plotted in Fig. 1.   It will be seen that the 5-methyl xylose shows a single very rapid reaction, as would be expected from a pentose substituted in position (5) so that only the γ-glycoside could be formed.   The 3-methyl xylose, on the other hand, shows two distinct reactions, and is in fact very similar to unsubstituted xylose.   For ready comparison the curve previously published for xylose is included.

[5] Levene, P. A., Raymond, A. L., and Dillon, R. T., *J. Biol. Chem.*, **95**, 699 (1932).

[6] By mistake, the sugar concentration is given in the paper cited in footnote 5 as 0.344 M.   It should be 0 0344 M

PHOSPHORIC ESTERS OF XYLOSE AND OF 5-METHYL MONOACETONE XYLOSE. THEIR BEARING ON THE NATURE OF THE PENTOSE OF YEAST NUCLEIC ACID

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The primary nature of *d*-ribose as a constituent of nucleic acid was questioned by Robinson[1] who pointed out the possibility of its formation, through Walden inversion, in the process of dephosphorylation of xylose-3-phosphoric acid. Robinson, in fact, suggested that a similar process may be responsible for the formation in nature of other hexoses, as for instance, galactose from glucose.

In the case of the ribose originally isolated by Levene and Jacobs[2] in their nucleic acid studies, the suggestion of Robinson does not apply for the ribose was derived from inosinic acid in which the phosphoric acid group is attached to the terminal, non-asymmetric carbon atom. However, the possibility that Walden inversion of xylose-3-phosphoric acid may take place is not excluded, and it was planned to test the possibility experimentally.

It was expected that phosphorylation of the previously prepared 5-acetyl and 5-benzoyl monoacetone xylose[3] would afford a convenient starting material for the preparation of 3-phospho-xylose. This phosphorylation was therefore performed. After removal of the acyl and acetone groups by hydrolysis, pentose phosphates were isolated but in small yield. Contrary to expectations, these phosphoric esters on treatment with methyl alcohol containing hydrogen chloride formed only the γ-glycosides.

[1] Robinson, R., *Nature*, **120**, 44 (1927).

[2] Levene, P. A., and Jacobs, W. A., *Ber. chem. Ges.*, **42**, 1198 (1909).

[3] Levene, P. A., and Raymond, A. L., *J. Biol. Chem.*, **102**, 317 (1933).

As this behavior is characteristic of pentoses substituted in position (5), xylose 5-phosphate was prepared for comparison. Monoacetone xylose was phosphorylated directly and a phosphoric ester was again obtained, this time in better yield. The rotation of the barium and sodium salts, and of the latter in borax solution agreed quite well with the corresponding rotations of the esters prepared by the first procedure. In addition, the rate of phosphate hydrolysis was nearly the same for the two products. Glycoside experiments again resulted in the formation of only the furanoside. Thus, xylose 5-phosphate is apparently formed from 5-acetyl and 5-benzoyl monoacetone xylose, presumably as the result of a migration at some stage of the reaction.

In order to avoid this complication, the previously described 5-methyl monoacetone xylose[4] was phosphorylated and the ester was obtained in fair yield. This phosphoric ester was next dephosphorylated by heating with barium methylate in methyl alcohol. From the reaction product there was isolated 5-methyl monoacetone xylose, identical with that used as starting material. Moreover, although the yield was but 47 per cent of the theoretical, no isomeric substance of similar nature was found in the mother liquors of the 5-methyl xylose. Thus we may conclude that either there was no Walden inversion or else there was a double one, once during phosphorylation, and once during hydrolysis. Of these possibilities, the one suggesting the absence of Walden inversion in these reactions seems the more probable.

It was hoped to prepare the 3-phosphoxylose through 5-trityl xylose. It was found, however, that the preparation of 5-trityl xylose in pure state is a troublesome process and work on its preparation is still in progress.

EXPERIMENTAL

Xylose Phosphoric Acid from 5-Acetyl Monoacetone Xylose—8 gm. of 5-acetyl monoacetone xylose[3] were dissolved in 80 cc. of dry pyridine cooled in ice-salt, and stirred mechanically. A solution of 3.4 cc. (1.1 mols) of distilled phosphorus oxychloride in 20 cc. of chloroform was dropped in over a period of 20 minutes. The mixture was kept at room temperature for 3 hours, and water

[4] Levene, P. A., and Raymond, A. L., *J. Biol. Chem.*, **102**, 331 (1933).

was then cautiously added. The mixture was concentrated under reduced pressure to remove most of the pyridine and was then extracted with chloroform until additional extracts contained no organic phosphorus. The chloroform solutions gave only the faintest test for halide. They were concentrated under reduced pressure, and water was added to produce an aqueous solution of the ester. A saturated barium hydroxide solution was added until the pH was 7.2 and the solution was again concentrated under reduced pressure to remove the liberated pyridine. The pH was again adjusted to 7.2 with barium hydroxide, and the concentration was continued. The product was finally dried over phosphorus pentoxide in a desiccator, powdered, and redried to constant weight. The yield was 10.2 gm. The composition agreed only fairly well with that of a phospho acetyl mono-acetone pentose.

5.460 mg. substance: 6.602 mg. CO_2 and 2.520 mg. H_2O
10.695 "   "   : 14.03 " $BaSO_4$
3.255 "   "   : 15.075 " ammonium phosphomolybdate (Pregl)

The analyses were calculated on a barium-free basis.

$C_{10}H_{17}O_9P$. Calculated. C 38.44, H 5.49, P 9.95
312.2   Found.   " 38.27, " 6.21, " 7.81

In an acetyl determination 0.200 gm. of dry barium salt required 4.88 cc. of 0.1 N sodium hydroxide. Calculated on a barium-free basis this becomes 5.66 cc., while the theoretical value is 6.41 cc.

Without further attempt at purification this material was subjected to acid hydrolysis to remove the acetone and acetyl groups. 5 gm. were dissolved in 50 cc. of 2 N sulfuric acid and heated at 80° for 2 hours. Without filtering the mixture was distilled under reduced pressure (with occasional addition of water) until no more acid appeared in the distillate. The undistilled residue was made alkaline (pH 8.3) with barium hydroxide, centrifuged to remove most of the barium sulfate, and the solution was filtered with charcoal. The filtrate was concentrated under reduced pressure to about 50 cc. and precipitated with an equal volume of 95 per cent alcohol. The precipitate was centrifuged off, washed with 50 per cent alcohol, and redissolved in water. The solution

was again concentrated to 50 cc. volume, reprecipitated, washed, and dried. The yield was 1.5 gm. The composition agreed with that of the barium salt of a pentose phosphate.

5.210 mg. substance: 3.105 mg. CO_2 and 1.340 mg. H_2O
10.690 "   "   : 6.615 " $BaSO_4$
 4.400 "   "   : 26.010 " ammonium phosphomolybdate (Pregl)
     $C_5H_9O_8PBa.$ Calculated. C 16.42, H 2.48, P 8.50, Ba 37.59
     365.5   Found.   " 16.25, " 2.88, " 8.58, " 36.41

The barium salt was converted, with sodium carbonate, to the sodium salt.

The rotation of the latter, in water, was $[\alpha]_D^{22} = \dfrac{+0.92° \times 100}{2 \times 3.74} =$ $+12.3°$ and the rotation in a half saturated borax solution was $[\alpha]_D^{22} = \dfrac{+0.56° \times 100}{2 \times 1.87} = +15.0°$.

Xylose Phosphoric Acid from 5-Benzoyl Monoacetone Xylose - 20 gm. of 5-benzoyl monoacetone xylose[3] were dissolved in 150 cc. of dry pyridine, cooled in ice, and stirred mechanically. A solution of 6.9 cc. (1.15 mols) of distilled phosphorus oxychloride in 40 cc. of chloroform was dropped in over a period of 20 minutes. The mixture was allowed to stand at room temperature for 3 hours and water was then cautiously added, with cooling, until no more heat was evolved. The mixture was concentrated under reduced pressure to remove most of the pyridine and was then taken up in chloroform. Considerable water was added and the aqueous layer was repeatedly extracted with chloroform until further extracts contained but little organic phosphorus. The combined chloroform extracts contained a trace of halide and were therefore washed with a very small quantity of water. The chloroform extract was concentrated under reduced pressure to a thin syrup and diluted with water. Barium hydroxide solution was added to pH 7.2 as for the acetyl compound and the solution was filtered with charcoal. It was again concentrated under reduced pressure to a thin syrup and this was taken up in about 400 cc. of acetone and precipitated with several volumes of pentane. The precipitate, which was a sticky mass, was redissolved in acetone and reprecipitated with pentane. This was repeated once more and the product was a flocculent white precipitate

which had a tendency to become gummy. It was dried over calcium chloride under reduced pressure, and then over phosphorus pentoxide to constant weight. The yield (including material obtained from the mother liquors by precipitations similar to those above) was 22 gm. The thoroughly dry material was insoluble in water as well as in acetone. The composition agreed approximately with that of a benzoyl monoacetone pentose phosphate.

5.126 mg. substance: 6.660 mg. CO_2 and 1.710 mg. H_2O
14.520 "   "   : 4.600 " $BaSO_4$
5.505 "   "   : 25.125 "   ammonium phosphomolybdate (Pregl)

The analyses were calculated on a barium-free basis.

$C_{15}H_{19}O_9P$.   Calculated.   C 48.10, H 5.12, P 8.30
374.2   Found.   " 43.40, " 4.57, " 8.13

5 gm. of this barium salt were dissolved in 50 cc. of dry methyl alcohol and 1.0 cc. of a 1.7 N solution of barium oxide in methyl alcohol was added. After $\frac{1}{2}$ hour at room temperature the solution was no longer alkaline to phenolphthalein and an additional 1.0 cc. of the alkali solution was added. After standing overnight, 200 cc. of water were added and the mixture was distilled under reduced pressure to remove the methyl benzoate and methyl alcohol. A precipitate (0.8 gm.) formed and was filtered off. The filtrate was concentrated under reduced pressure to a volume of 130 cc. and 32 cc. of 5 N sulfuric acid were added. The mixture was heated at 80° for 3 hours and was then cooled and neutralized (pH 7.5 to 8) with barium hydroxide. The precipitate was centrifuged off and washed once by centrifugation. The combined solutions were concentrated under reduced pressure, filtered with charcoal, and precipitated with an equal volume of 95 per cent alcohol. The precipitate was washed with 50 per cent alcohol, redissolved in water, and reprecipitated as before. The product was filtered off, washed with 50 per cent and then 95 per cent alcohol, and dried to constant weight. The yield was 0.8 gm.

The rotation of the barium salt, in water, was $[\alpha]_D^{20} = \dfrac{+0.23° \times 100}{1 \times 3.0} = +7.7°$. The sodium salt, prepared from the barium salt with a solution of sodium carbonate, had a rotation,

in water, of $[\alpha]_D^{22} = \dfrac{+0.32° \times 100}{2 \times 5.0} = +3.2°$, and in half saturated

borax solution of $[\alpha]_D^{22} = \dfrac{+0.20° \times 100}{2 \times 2.5} = +4.0°$.

> 12.005 mg. substance: 6.900 mg. BaSO₄
>   4.894 "   "   : 30.050 " ammonium phosphomolybdate (Pregl)
>   C₅H₉O₈PBa.   Calculated.   Ba 37.59, P 8.58
>   365.5   Found.   " 33.82, " 8.92

Xylose Phosphoric Acid from Monoacetone Xylose—20 gm. of crystalline monoacetone xylose were dissolved in 125 cc. of dry pyridine and cooled to −30°. A solution of 10.3 cc. of phosphorus oxychloride (1.1 mols) in 25 cc. of chloroform was added in several portions with vigorous stirring. The mixture was transferred to an ice-salt bath for 2 hours and water was then added in small portions and with careful cooling. The mixture was made alkaline with barium hydroxide, and concentrated under reduced pressure, with occasional addition of water, until the pyridine was removed. The solution was made 2 N with sulfuric acid and was heated at 80° for 2 hours. The chloride was removed from the acid solution with freshly prepared silver sulfate; the filtrate was saturated with hydrogen sulfide and then aerated to remove the excess. Barium hydroxide was added to pH 8 and the filtrate was concentrated under reduced pressure to a small volume. The product was precipitated by addition of 2 to 3 volumes of 95 per cent alcohol and the precipitate was washed with 50 per cent alcohol. It was redissolved in water, filtered with charcoal, reprecipitated as above, and washed with 50 per cent and then with 95 per cent alcohol. The product was dried to constant weight. The yield was only 5 to 5.5 gm. The barium salt had a rotation, in water, of $[\alpha]_D^{22} = \dfrac{+0.20° \times 100}{2 \times 2.0} = +5.0°$. The sodium salt, prepared from the barium salt by addition of a solution of sodium carbonate, had a rotation in water of $[\alpha]_D^{22} = \dfrac{+0.32° \times 100}{2 \times 5.0} = +3.2°$ and in half saturated borax, of $[\alpha]_D^{22} = \dfrac{+0.24° \times 100}{2 \times 2.5} = +4.8°$.

Phosphorylation of 5-Methyl-1,2-Monoacetone Xylose—To 90 cc. of cold (−40°) dry pyridine there were added 7.2 cc. (1.1 mols) of distilled phosphorus oxychloride, and then in one portion, a solution of 15 gm. (1.0 mol) of pure 5-methyl monoacetone xylose[4] in 60 cc. of cold (−30°) dry pyridine. The mixture was vigorously stirred and then after a few minutes was transferred to an ice-salt bath for 3 hours. Water was added in small portions with careful cooling and a solution of barium hydroxide was then added to a pH of 8. The mixture was concentrated under reduced pressure to remove all the pyridine, water being added from time to time. The solution was filtered with charcoal and concentrated further to a volume of about 100 cc. 4 volumes of acetone were added and the barium chloride was filtered off. The barium chloride precipitate was extracted three times with boiling 90 per cent acetone and the extracts and original solution were combined and concentrated under reduced pressure to a volume of 50 to 60 cc. 4 to 5 volumes of acetone were added and the mixture was cooled in the ice box. The small precipitate was removed by filtration with charcoal. The solution still contained a trace of halide and this was removed by distilling off the acetone, adding water, and shaking for several minutes with freshly prepared silver sulfate. The filtrate, which was turbid, was concentrated to a thin syrup which was taken up in acetone. The acetone solution was filtered with charcoal and was then clear and almost water-white.

The acetone solution was carefully precipitated with several volumes of absolute ether giving a white, non-hygroscopic powder which was filtered off and washed with more ether. From the filtrate additional product was obtained by concentrating to a small volume and precipitating in the same way. From the second filtrate still further material was obtained by drying over phosphorus pentoxide under reduced pressure. This final material was extracted with boiling ether to remove any unchanged methyl monoacetone xylose which it might have contained. The yield of combined material was 20 gm. It was very faintly reducing before acid hydrolysis and strongly reducing afterwards. The composition agreed fairly well with that of the acid-barium salt of a methyl monoacetone pentose phosphate.

0.100 gm. substance: 0.0384 gm. BaSO$_4$
6.834 mg.   "   : 42.920 mg. ammonium phosphomolybdate (Pregl)
  (C$_9$H$_{16}$O$_8$P)$_2$Ba.   Calculated.   Ba 19.52, P 8.82
  703.7   Found.   " 22.59 " 9.12

The rotation of the substance, in water, was $[\alpha]_D^{22} =$

$$\frac{+0.16° \times 100}{2 \times 2.0} = +4.0°.$$

Dephosphorylation of 3-Phospho 5-Methyl Monoacetone Xylose—
5 gm. of the barium salt of 3-phospho 5-methyl monoacetone
xylose were mixed with 15 cc. of methyl alcohol and 25 cc. of a
1.7 N solution of barium oxide in methyl alcohol, and sealed in a
Pyrex flask body. The mixture was shaken until the salt dis-
solved and was then heated in a boiling water bath for 20 hours.
The tube was cooled and opened, 1 cc. of water was added, and
the mixture was concentrated to dryness under reduced pressure.
The solid was extracted several times with boiling ether, the
extracts were concentrated under reduced pressure, filtered with
charcoal, concentrated further, and cooled in the ice box. Pen-
tane was added just to turbidity and the product crystallized.
The yield, in three crops, was 1.4 gm., or 47 per cent of the theo-
retical. Less than 0.1 gm. of syrup remained from the ether
extract so that apparently no isomeric product was formed at
the same time. The crystalline material was recrystallized from
ether-pentane. A rotation, melting point, and mixed melting
point showed it to be identical with the 5-methyl monoacetone
xylose used as starting material for the phosphorylation.

Rate of Phosphate Hydrolysis in Xylose Phosphates—Solutions
of the barium salts of the phospho esters containing about 1 mg.
of phosphorus per cc. were made 1 N with hydrochloric acid,
sealed in Pyrex tubes, and heated in the steam bath. At the
desired times the tubes were removed, cooled, and opened.
Samples were diluted exactly and the inorganic phosphoric acid
was determined colorimetrically by the Kuttner-Cohen[5] method.
The results are given in Table I from which it will be seen that
the ester from monoacetone xylose and that from the 5-benzoyl
monoacetone xylose have substantially the same rates of phos-
phate hydrolysis.

[5] Kuttner, T., and Cohen, H. R., *J. Biol. Chem.*, **75**, 517 (1927).

Glycoside Formation of Xylose Phosphates—0.0344 M solutions of the barium salts of the phospho esters in methyl alcohol containing 0.5 per cent free hydrogen chloride were measured at room temperature and at 50°.   The esters used were those from monoacetone xylose and from 5-benzoyl monoacetone xylose. The rotations were measured in 2 dm. tubes at 25° with sodium D light.   It was found that a small negative change in rotation occurred with each ester at room temperature and no further

TABLE I

Rate of Phosphate Hydrolysis

| Xylose phosphate from monoacetone xylose | | Xylose phosphate from 5-benzoyl monoacetone xylose | |
Time	Hydrolysis	Time	Hydrolysis
hrs	*per cent*	*hrs*	*per cent*
0 0	0 2	0 0	0 0
0 5	23	0 5	29
1 5	51	1	44
3	71	2 5	70
6	93	5	86
12	97		

TABLE II

Reduction Determination during Glycoside Formation at Room Temperature. Phospho Ester from 5-Benzoyl Monoacetone Xylose

Time	Reduction	Time	Reduction
hrs	*per cent*	*hrs.*	*per cent*
0	93	24	9
1	25	48	4
4	14		

change at 50° in 24 hours.   However, the magnitude of the changes ($[\alpha]_D^{25} = 16° \rightarrow 8°$) was so small that it was not considered sufficient evidence for formation of the γ-glycoside, and it was therefore substantiated by reduction determinations.   The micro-Willstätter technique previously developed[6] was used and the results are given in Table II.   It will be seen that the free sugar disappears rapidly and in fashion characteristic of γ-glycoside formation.

[6] Levene, P. A., Raymond, A. L., and Dillon, R. T., *J. Biol. Chem.*, **96**, 449 (1932).

THE RELATION OF IRON AND COPPER TO THE RETIC-
ULOCYTE RESPONSE IN ANEMIC RATS*

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The phenomenon of reticulosis has been used extensively as a
clinical indication of the activity of the hematopoietic organs
since 1898 when Poggi (1) observed a rapid increase in the number
of reticulocytes during blood regeneration in cases of anemia and a
subsequent decrease when the erythrocyte count approached
normal values. The rate of reticulocyte response has been found
to be of distinct value in pernicious anemia studies. In 1928
Minot, Murphy, and Stetson (2) observed a response of reticulo-
cytes, similar to that obtained in pernicious anemia, in cases of
secondary anemia treated with liver. More recently Minot and
his associates (3, 4), and Yang and Keefer (5) found a positive
reticulocyte response in human cases of secondary anemia treated
with iron. Although the factors affecting the production of reticu-
locytes during the treatment of pernicious anemia in humans have
been investigated quite thoroughly, the mechanism of reticulocyte
response during the treatment of secondary anemia has received
little attention.

Very little is known about the reticulocyte changes in the blood
of rats recovering from nutritional anemia. Much valuable
information should be gained from studies with the rat because
the requirements for hemoglobin formation are so well established.
Beard, Baker, and Myers (6) found that iron alone, when fed to
anemic rats subsisting on a milk diet, stimulated reticulocyte

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formation. When elements such as Cu, Mn, As, Ge, etc. were fed together with iron they found no greater degree of reticulosis but the rate of maturation of the erythrocytes was increased. Since the workers in Myers laboratory (7) have always obtained hemoglobin regeneration with iron alone, it is difficult to ascertain the action of copper from their work. The following studies were inaugurated to determine the exact relation of iron and copper to reticulocyte response in carefully controlled anemic rats.

All animals were made anemic by the usual procedure (8). Supplements were not administered until a very severe degree of anemia was attained (2 to 3 gm. of Hb per 100 cc.). The supplements were in most cases given in the milk which constituted the basal diet. Hemoglobin determinations as well as erythrocyte and reticulocyte counts were made daily. For the latter purpose the method of Friedländer and Wiedemer (9) proved to be satisfactory in spite of its limitations (10). The reticulocytes were recorded in absolute numbers per c. mm. of blood.

The reticulocyte content of the blood of anemic rats shows some variation; the values, however, are seldom above 500,000 per c. mm. and generally fall between 200,000 and 400,000.

Chart 1 shows the daily changes in the hemoglobin, erythrocyte, and reticulocyte content of the blood of an anemic rat given 1 mg. of Fe and 0.1 mg. of Cu daily. Under optimum conditions for recovery from nutritional anemia, i.e. when adequate amounts of both iron and copper are fed, the increase in the number of reticulocytes is very rapid. A maximum of 1,500,000 to 1,700,000 per c. mm. is attained in 4 to 5 days after the mineral additions are started. This increase is followed by an almost equally rapid decrease to values between 200,000 to 400,000. In the meantime the hemoglobin and erythrocytes show a rapid and continued increase. The hemoglobin reaches values of approximately 8 gm. per 100 cc. before the number of reticulocytes begins to decrease. This response may be considered typical for our experimental conditions because similar results have been obtained with a large number of animals.

When iron alone was fed to anemic rats, the reticulocytes, erythrocytes, and hemoglobin showed no appreciable change.

The results for one of the rats fed iron alone are given in Chart 2. After this rat had received 0.5 mg. of iron daily for 29 days, 0.1 mg. of Cu was substituted for the iron.   As soon as copper was supplied a response very similar to that shown in Chart 1 was observed.   The increase in hemoglobin was not maintained in this case because the store of iron was soon depleted.   These results clearly demonstrate that iron alone under the conditions of our
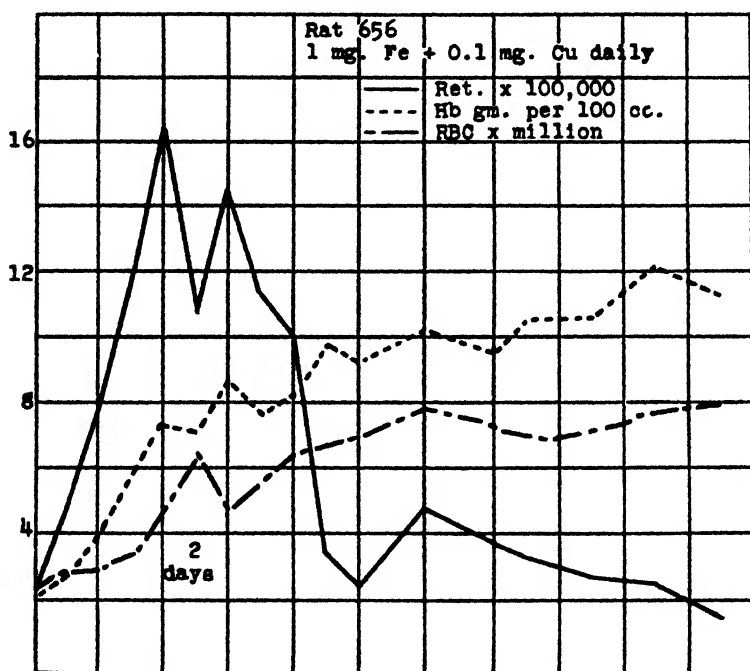
CHART 1. Daily changes in the hemoglobin, erythrocyte, and reticulocyte content of the blood of an anemic rat fed 1 mg. of iron and 0.1 mg. of copper daily.

experiments cannot stimulate reticulocyte formation.   When a supply of both iron and copper is available in the animal the typical response occurs.

The effect of feeding copper alone is illustrated in Chart 3. A definite reticulocyte response was observed in all anemic rats given 0.1 mg. of Cu daily.   The shape of the reticulocyte curve, however, is distinctly different from that observed in the case of a
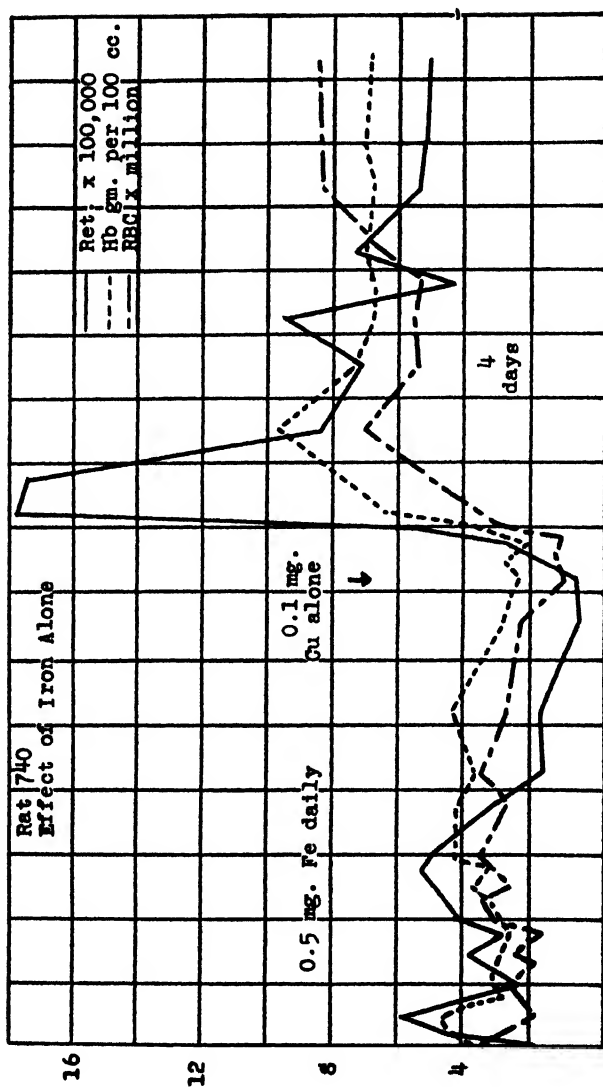
CHART 2. Blood changes in a rat receiving iron alone. After 29 days of iron therapy copper was fed instead of the iron.
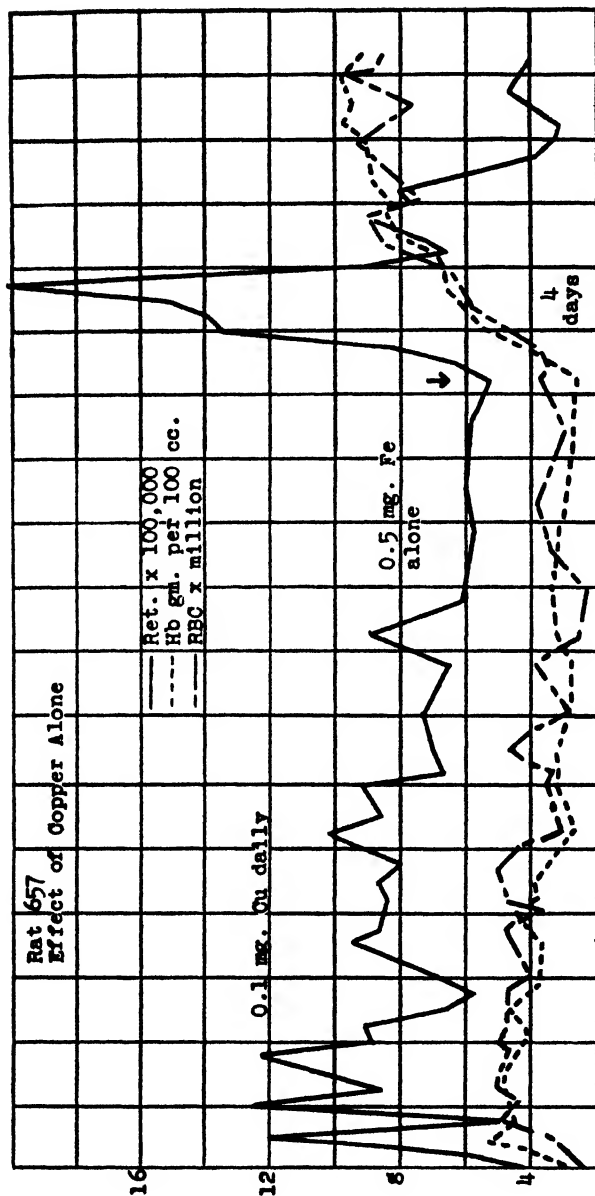
CHART 3. Blood changes in a rat receiving copper alone. The copper was replaced by iron alone after the rat had been on experiment for 49 days.

typical response. It is characterized by daily variations in the count as well as by a prolonged persistence of the reticulocyte values above the limits observed in untreated anemic animals. In the case of Rat 657 the higher level was maintained for 49 days as long as copper alone was supplied, but as soon as 0.5 mg. of Fe was fed in place of the copper a typical response was obtained and the level returned to normal. It is evident from the curves in Chart 3 that there occurred a slight initial rise of hemoglobin and erythrocytes with copper alone. This suggests that even severely anemic rats contain a small amount of iron which may be used for hemoglobin building in the presence of adequate amounts of copper. The very small increase in hemoglobin demonstrates that the anemic rats used in our laboratory are extremely depleted in iron reserves.

The effect of smaller amounts of copper fed with sufficient amounts of iron (0.5 mg.) is shown in Chart 4. Typical responses were obtained with levels down to 0.005 mg. of Cu. At this level the duration of the reticulosis was somewhat prolonged and the rate of hemoglobin and erythrocyte regeneration was much slower. With 0.001 mg. of Cu the hemoglobin formation was retarded and the reticulocyte response was much prolonged.

The results with feeding of smaller amounts of copper were more variable, probably owing to differences in the degree of copper depletion in the rats before the mineral supplements were added. Chart 5 shows the results when different levels were used for the same animal. In this case a slight temporary response was observed with 0.0005 mg. of Cu plus 0.5 mg. of Fe, but this response was undoubtedly due to a supplementary effect of small amounts of copper present in the animal. This reserve was soon used up because the hemoglobin and erythrocytes failed to increase appreciably. After the depletion period the addition of 0.001 mg. of Cu produced only a small response. When the copper intake was increased to 0.005 mg. the response was practically identical with that shown for this amount of copper in Chart 4. It is difficult to fix a definite minimum level of copper, which in the presence of sufficient iron will produce a typical response, but our results indicate that the level lies between 0.005 mg. and 0.01 mg. daily.

The curves in Chart 6 indicate in a similar manner that in the
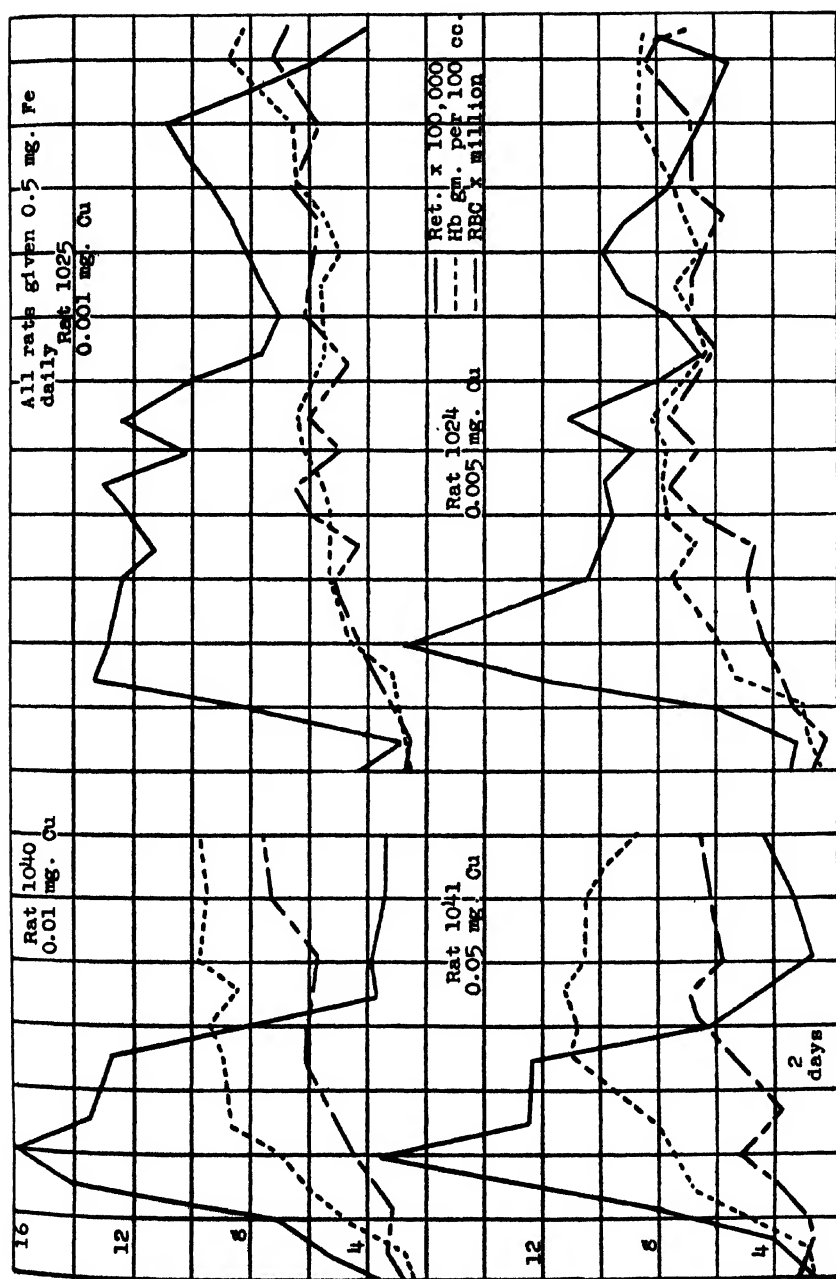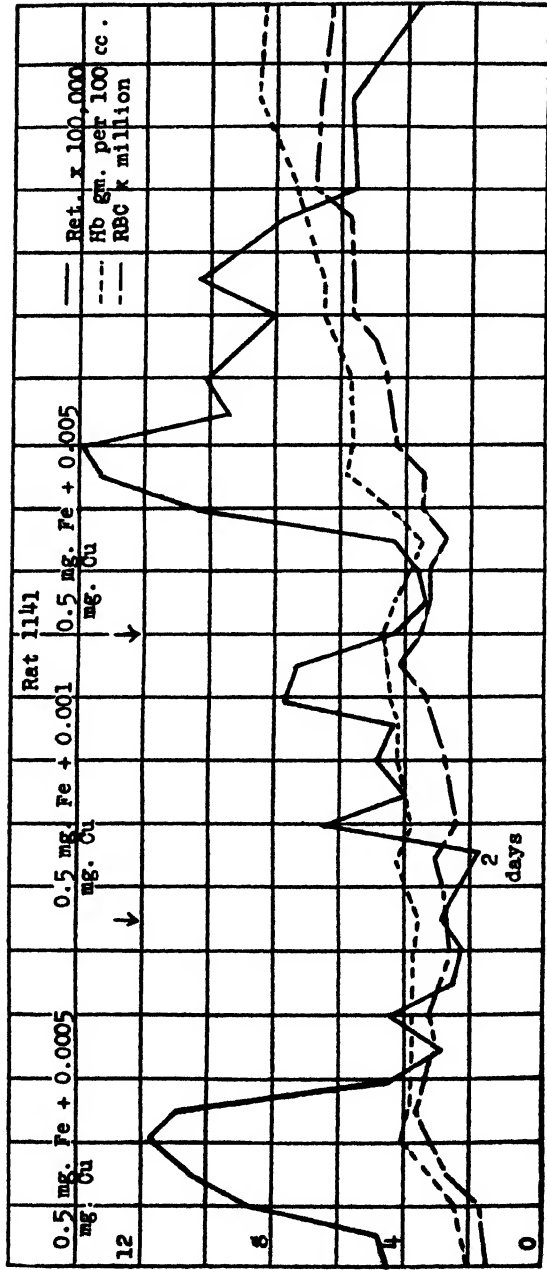
CHART 5. The effect of suboptimal amounts of copper on the reticulocyte response in the presence of adequate amounts of iron

presence of adequate quantities of copper the anemic rat requires at least 0.3 mg. of iron daily for the production of a typical reticulocyte response. The response in the presence of 0.2 or 0.1 mg. of iron was more prolonged. The results for the rat receiving only 0.04 mg. of Fe are very similar to those obtained with many of the animals fed copper alone. When some response is obtained with such minute quantities of iron it is easy to see why a response may be observed with copper alone. Even the most anemic animal may contain iron which can be mobilized for hemoglobin formation in the presence of copper.

The fact that both iron and copper are necessary to produce marked reticulosis in anemic rats, and that these elements are also essential for hemoglobin formation suggests an intimate relationship between the two processes. In order to study this relationship further the effect of hemoglobin injections on the reticulocyte content of the blood of anemic rats was studied. Since it is very difficult to prepare any quantity of pure undenatured hemoglobin, blood preparations were made according to the method of Whipple and Robscheit-Robbins (11). The hemoglobin solutions were prepared from rat blood and 2 cc. containing 0.08 gm. of hemoglobin were injected intraperitoneally into anemic rats every other day. No ill effects from the injections were observed in any case.

The blood changes for one of the four rats that received this treatment are given in Chart 7. It is readily seen that the reticulocyte response is very similar to that obtained when suboptimal amounts of iron and copper were fed. The reticulocytes remained at a high level as long as the injections were continued and the hemoglobin and erythrocytes increased very slowly. These results indicate that the presence of added hemoglobin in the blood was sufficient to stimulate reticulosis but the quantity was not large enough to build up the hemoglobin content of the blood and consequently the reticulocytes remained at a high value. It is difficult to establish definitely whether the active substance is actually hemoglobin or some other constituent of the blood preparation. Oka (12) working with rabbits suffering from posthemorrhagic anemia reports a similar conclusion.

Through the work of Minot and his associates it is definitely established that liver preparations which are active in the cure of
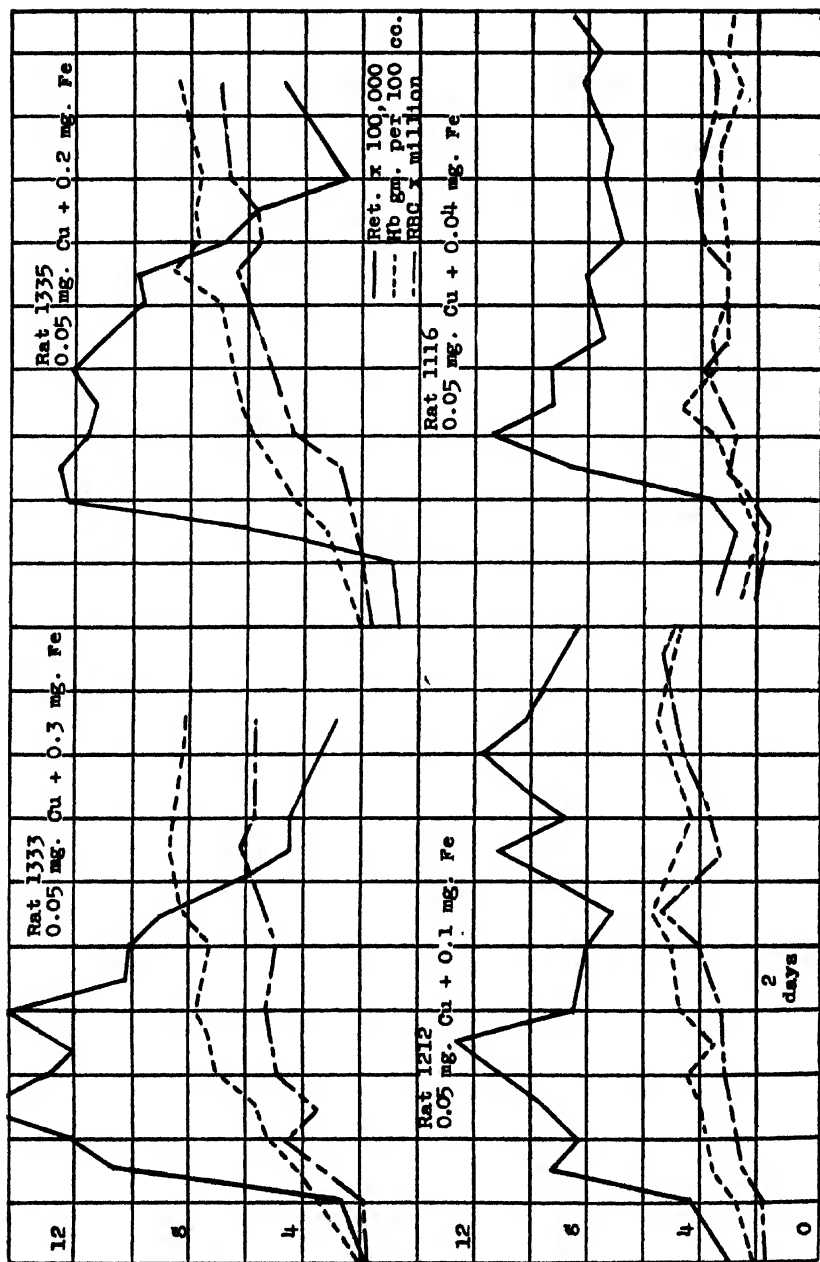
CHART 6. Reticulocyte response with varying amounts of iron in the presence of adequate quantities of copper.
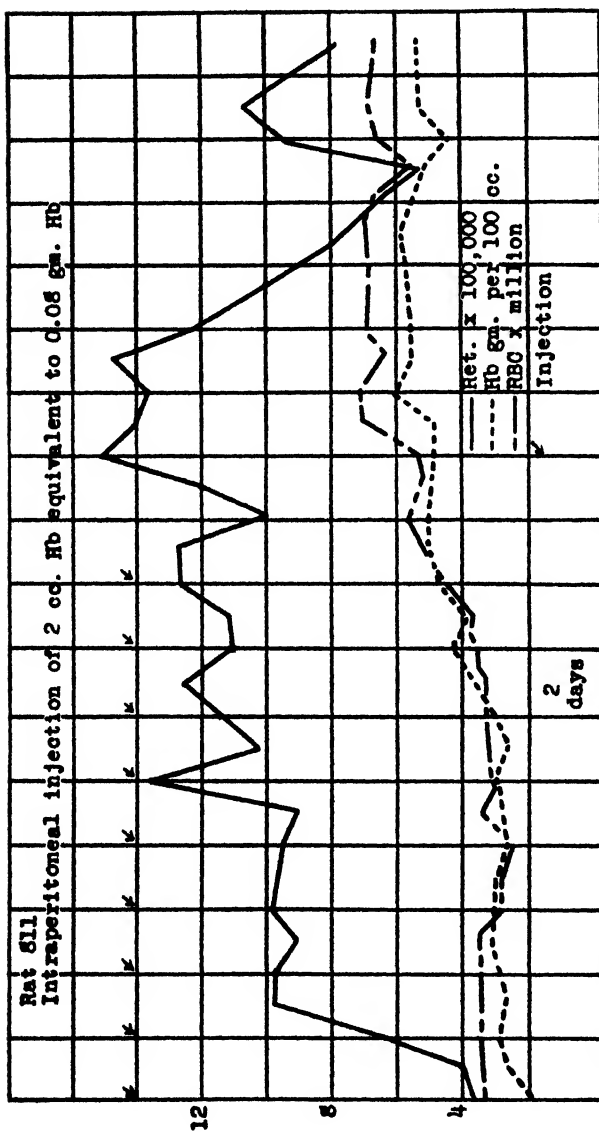
CHART 7. Blood changes during intraperitoneal injections of hemoglobin solutions

pernicious anemia produce a reticulocyte response in humans.   A large percentage of the cases of secondary anemia fail to respond to the feeding of pernicious anemia fractions.   The question therefore arises: does a reticulocyte response occur without hemoglobin formation when secondary anemias are treated with pernicious anemia fractions or are these fractions unable to stimulate reticulosis in the absence of hemoglobin regeneration?   If the suggestion that rapid hemoglobin formation initiates a reticulocyte response during the treatment of secondary anemia is correct, then a pernicious anemia fraction low in iron and copper should have no effect on the reticulocytes in the blood of anemic rats.

Several liver extract preparations used for the treatment of pernicious anemia were fed to anemic rats.   Some were administered orally, and one preparation which was highly purified was injected intraperitoneally.   The unpurified preparation produced a small but definite response; however, when the material was freed from all or most of the copper the response was very slight.   This indicates that any stimulating effect which is obtained with liver extracts in this type of anemia is due mainly to the presence of iron and copper and that if these elements are removed liver preparations are unable to produce a typical reticulocyte response in secondary anemia.

<div align="center">DISCUSSION</div>

From the results presented it is evident that iron alone is equally inadequate for the production of reticulocytes as it is for the regeneration of hemoglobin in rats depleted of their stores of copper and rigorously denied access to appreciable amounts of this element.   Only when iron is supplemented by definite amounts of copper can a typical reticulocyte response be obtained.   This is opposed to the results of Beard, Baker, and Myers (6) working with rats, and in apparent contradiction to the observations made on humans by Minot and Heath (4), Josephs (13), and others. However, the relatively small amounts of copper which have been shown to be essential for stimulation of hematopoietic activity furnish a basis on which this apparent disagreement of results can be explained.   From Chart 4 it is evident that an intake of only 0.005 to 0.01 mg. of copper daily is necessary in the presence of sufficient iron to bring about a typical reticulocyte response,

and that still smaller amounts of copper may lead to increased numbers of reticulocytes. The fact that Beard, Baker, and Myers obtained a reticulocyte response with iron alone clearly demonstrates that their rats had some available copper with the iron. Whether the source of this copper was from the animal or from the milk we cannot say. Our results also indicate that more precautions must be taken to prevent reticulocyte response than are necessary to prevent appreciable hemoglobin regeneration when iron is fed alone. The rats used in our experiments ingested 0.007 to 0.008 mg. of Cu daily from the milk (50 cc. of milk containing 0.15 mg. of Cu per liter). It may be suggested that this amount of copper should produce a reticulocyte response since definite responses have been observed with 0.005 mg. of copper daily. The copper in the milk itself may not be as available as that added in the form of copper sulfate or that entering the milk from copper contamination.

We cannot agree with Beard, Baker, and Myers that copper acts by increasing the rate of maturation of the red blood cells. The decrease in reticulocyte concentration is not dependent upon copper *per se* but upon the amount of hemoglobin present in the blood.

Work with humans naturally cannot be controlled with the precision possible in animal experimentation. When iron alone is administered to humans suffering from secondary anemia, there is no way of estimating the store of available copper in the body, and it is impossible to attempt a depletion of this store before the iron is given. Anemic patients, therefore, may in many cases contain or ingest from the food sufficient copper to meet the requirements. Thus, it is easy to see why a typical reticulocyte response is believed to be dependent upon the addition of iron alone. The fact that an increase in hemoglobin is obtained in the human cases indicates that copper is available, for hemoglobin cannot be formed in the absence of this element. The complete mechanism of reticulosis can only be studied when carefully controlled animals are used.

Heath, Strauss, and Castle (14), suggest from their work with humans that there is a close correlation between the hemoglobin of the blood and reticulocyte response. We are in complete accord with this view. The rapid and large increases in reticulo-

cytes which we observed when optimum amounts of iron and copper were fed are undoubtedly due to the very low hemoglobin content in the blood of the rats used. A careful inspection of all our curves indicates that a slight increase in hemoglobin is generally observable whenever an increase in reticulocytes is obtained. This increase was not evident in every case but the amount of hemoglobin required may be so small that it is difficult to detect with the method used. There is also the possibility that the active material is not hemoglobin but a precursor which is dependent upon iron and copper for its formation. Cooke (15) has suggested that the stainable substance of reticulocytes is probably a hemoglobin compound. It is also evident from all the curves that the number of reticulocytes recedes to normal values only after the hemoglobin has reached values of about 8 gm. per 100 cc.

Cases of posthemorrhagic experimental anemia which show spontaneous remission form no exception to the work reported here. In these cases the elements necessary for hemoglobin formation are present in the animal and therefore reticulocyte response may be observed without ingestion of iron and copper.

The results obtained with liver preparations indicate that the active material in liver cannot produce reticulosis in all forms of anemia. In pernicious anemia the response is due to an organic factor present in liver, but in secondary anemias the response is dependent upon the ingestion of sufficient iron and copper for hemoglobin formation. It is very probable that even in pernicious anemia the reticulocyte response following liver therapy depends in part upon the presence of sufficient available iron and copper.

SUMMARY AND CONCLUSIONS

1. A typical reticulocyte response characterized by a very rapid increase in reticular red blood cells to a maximum of about 1,500,000 per c. mm. in 4 to 5 days and an almost equally rapid decrease to values between 200,000 to 400,000, together with a definite increase in hemoglobin and erythrocytes has been observed in a large number of severely anemic rats given adequate amounts of iron and copper.

2. Neither iron alone nor copper alone can produce a typical reticulocyte response in rats suffering from severe nutritional

anemia. Iron alone fails to initiate a response, while copper alone produces a small prolonged response. The results with copper alone are undoubtedly due to the action of the copper on small available supplies of iron in the body.

3. The minimum daily requirements for the production of a typical reticulocyte response in an anemic rat are approximately 0.3 mg. of iron and 0.005 to 0.01 mg. of copper.

4. The intraperitoneal injection of blood hemoglobin into anemic rats caused a reticulocyte response similar to that obtained with suboptimal amounts of iron and copper.

5. Pernicious anemia fractions low in iron and copper failed to produce a typical reticulocyte response.

6. The action of pernicious anemia factors and iron and copper in the treatment of pernicious and secondary anemias is discussed.

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OXIDATIONS BY ERYTHROCYTES AND THE CATALYTIC INFLUENCE OF METHYLENE BLUE

I. THE OXIDATION OF LACTATE TO PYRUVATE*

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The observations of Harrop and Barron (1, 2), that the addition of methylene blue to mammalian bloods or to suspensions of non-nucleated erythrocytes causes these cells to respire, with the oxidation of sugar or its derivatives, indicate that the lack of respiratory activity of these cells in their natural state is due to the absence of an oxygen-activating catalyst. When such a catalyst is supplied by adding methylene blue, these cells exhibit *in vitro* a seemingly physiological type of respiration, and appear to constitute exceptionally suitable material for analysis of factors concerned with cell respiration.

Soon after the appearance of Harrop and Barron's first papers Wendel and Shaffer began a study of the blood cell-methylene blue system and have published several preliminary reports (3, 4). In the meantime the subject has been investigated also by Zeile and von Euler (5), Michaelis and Salomon (6), Engelhardt (7), and especially by Warburg and associates (8-11), in whose hands it has been developed into an important chapter of biological oxidations.

The experiments to be reported now had two objects. First, we wanted to determine whether the carbohydrate oxidized in this system is glucose or an intermediate in the glycolytic reaction *before* lactic acid, or is lactate after its formation by the non-oxidative glycolysis. Secondly, we hoped to obtain a clue to the mech-

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* The data are taken from a dissertation presented to the Board of Graduate Studies in partial fulfilment of the requirements for the degree of Doctor of Philosophy, Washington University, June, 1932.

anism of the methylene blue catalysis. This question is considered in Paper II.

Methods

Fresh dog blood—in one instance rabbit blood—drawn without anesthetic was used. In some experiments defibrinated blood and in others erythrocytes, obtained by centrifugation of defibrinated blood and removal of the serum and buffy coat, were employed. Ridding the cells of sugar was accomplished either by washing with saline or by incubating the red cells (after removing the serum and white cells) at 37.5° for a period of 1 to $1\frac{1}{2}$ hours. The latter procedure provides what we call *glycolyzed cells*. The cells were suspended in an equal volume of freshly prepared nearly isotonic solution of the following composition: 0.1 M sodium chloride, 0.04 M sodium phosphate (pH 7.4), and 0.02 to 0.01 M sodium *dl*-lactate (pH 7.4). U.S.P., medicinal, methylene blue (from the National Aniline and Chemical Company, Inc.) was used. Where the dye concentration is not stated in the text or tables, it is 1.3×10^{-4} M.

The blood (or corpuscle suspension) was incubated with gentle rocking at 37.0–37.5°. Oxygen consumption and CO_2 production were determined (by the manometric methods of Van Slyke and Neill (12) on 1 or 2 cc. samples) as changes in content of the cell suspensions following incubation in completely filled heavy walled test-tubes which were stoppered with 1-hole rubber stoppers bearing capillary tubes (10 to 12 inches long). When gas changes were not measured the cell suspensions were incubated in stoppered 300 cc. Erlenmeyer flasks. Glass beads were placed in each tube to prevent sedimentation.

Sugar was determined in duplicate on 5.0 cc. samples of zinc sulfate filtrates (Somogyi (13)) with KI-free Shaffer-Hartmann reagents (14). In a few experiments in which $CaSO_3$ was added to the blood, interference with the sugar determinations was avoided by oxidizing the small amount of sulfite in the deproteinized filtrate with iodine before mixing with the sugar reagent. Here a copper reagent containing KI (15) was used. The sugar values are significant to about 0.1 mM per liter.

Lactic acid and pyruvic acid were determined—the latter after reduction to lactic acid (16)—in duplicate by the Friedemann-Cotonio-Shaffer method, modified as to acid reagent (16), on 20.0

cc. samples of 1:10 mercuric chloride filtrates after treatment with $CuSO_4$ and lime. With this quantity of filtrate differences greater than about 0.2 mM per liter are significant.

Acetaldehyde was determined on 10.0 cc. samples of precipitated blood by aeration with reflux distillation (with an excess of $CaCO_3$) into bisulfite, as in the lactic acid determinations. The bound bisulfite was estimated in the usual manner. The method gave 90 per cent recovery with small quantities of acetaldehyde.

Results

Our results with dog blood and erythrocytes (Table I) confirm the observation of Barron and Harrop that the addition of methylene blue brings about a decrease of lactic acid formation, while the rate of sugar disappearance remains the same or is somewhat increased. Indigo carmine and blood hematin are practically without effect, possibly because of non-diffusion into the cells. The absence of effect with blood hematin is in agreement with the observations of Warburg and Kubowitz (10).

Lactate Oxidation—When glucose is first removed and lactate added, either by glycolysis (Experiments 128 and 150 of Table II) or by addition of lactate to washed cells (Experiments 157-a and 151-b) a similar disappearance of lactate occurs on incubation with the dye. In the absence of the dye, lactate increases slightly, perhaps from hexosephosphate, since little or no glucose was present.

Pyruvate Formation—Table II shows a summary of experiments in which pyruvic acid also was determined. In some experiments there is evidence of slight pyruvic acid formation in the controls without methylene blue. In the presence of the dye the amount formed is much greater.

From the data in Table II we may draw up a balance (Table III) in order to compare the loss of glucose plus lactate with the pyruvate formed in the presence of methylene blue. It will be seen that with glycolyzed or washed erythrocytes, practically free from glucose but containing lactate, there is moderate agreement between loss of lactate and gain of pyruvate. This indicates the oxidation of lactate only to pyruvate. Added pyruvate is not acted upon by normal dog erythrocytes in the presence or absence of methylene blue, nor by reticulated erythrocytes (dog), the

normal respiratory activity of which is large. With defibrinated blood or erythrocytes and glucose, on the other hand, more carbohydrate disappears than is accounted for by lactate or pyruvate. It may be concluded, therefore, that the glucose which disappears in the presence of methylene blue has at least two fates, one via

TABLE I

Effect of Methylene Blue on Glycolysis and Lactic Acid Production (Incubation Period, 3 Hours)

The results are expressed in mM per liter of suspension.

	Experiment No	Glucose			Lactate			Total loss of sugar + lactate as mM glucose	
		Initial	Decrease		Initial	Increase			
			Control	Methylene blue		Control	Methylene blue	Control	Methylene blue
Defibrinated dog blood	23*	6 0	2.7	1 6	1 8	4 8	0.4	0 3	1.4
	24*	6 6	3 3	2.5	2 1	5.7	1.1	0.45	2.0
	66-b	4.4	3.6	3.4	4 0	5 6	1 6	0 8	2 6
	67-a	2.4	2 1	2.1	4.8	3 3	0.4	0 45	1.9
	148	8.1	3 7	4.0	2.9	5.5	3 7	0.95	2 15
	73	8.5	3.2	3.4	1.8	6.4	3 5	0	1.7
	73†			(3.4)			(5 9)		(0.4)
	73‡			(3.5)			(6.2)		(0.4)
Washed erythrocytes with added glucose and lactate	69-c	4.7	2.1	2.3	12 8	2.3	−0.6	1.0	2 6
	0.005 per cent hematin			(2.0)			(2 2)		(0 9)
	0.01 per cent hematin			(1.7)			(1.6)		(0.9)

* Concentration of methylene blue = 0.7 mM per liter.

† 0.01 per cent indigo carmine instead of methylene blue.

‡ 0.05 per cent indigo carmine instead of methylene blue.

glycolysis to lactate and the oxidation of this to pyruvate, and the other not via lactate.

Oxygen Consumption and Methemoglobin Formation—Table IV shows the results of experiments in which the oxygen content and capacity, and CO_2 content, as well as the sugar, lactate, and pyruvate changes, were followed on the same blood cell suspen-

TABLE II
Formation of Pyruvate from Lactate Oxidation (mM per Liter)

	Experiment No.	Incubation	Glucose			Lactate			Pyruvate		
				De-crease			Change			Change	
			Initial	Control	Methylene blue	Initial	Control	Methylene blue	Initial	Control	Methylene blue
		hrs									
Defibrinated dog blood	148	3	8 1	3 7	4 0	2 9	+5 5	+3 7	0 1	+0 5	+2 3
	149	3	5 3	2 7	2 9	2 8	+4 6	+2 6	0 3	0	+2 0
	126-a	3½	8 2	4 3	5 1	2 7	+6 3	+4 0	0	0	+2 5
	221	3	10 1	4 5	5 3	3 6	+7.3	+6 4	0 2	0	+1 2
Erythrocytes in buffer + glucose	IV-2	3½	9 0	3 5	3 6	1 3	+5 2	+2 9	0 2	-0 1	+1 5
Glycolyzed erythrocytes	128	3	0 2			10 1	+0 9	-1 8	0	+0 6	+2 7
	150	3				11 6	0	-2 0	0	+1 0	+2 9
Washed erythrocytes	157-a	3				1 1	+0 7	-0 2	0 1	+0 4	+1 3
	151-b	3*				1 4	+0 5	+0 2	8 7	+0 5	+1 1
Reticulated erythrocytes + pyruvate	IV-5	3				1 0	+0 4		9 0	+0 3	

* Pyruvate added.

TABLE III
Comparison of Increase in Loss of Glucose Plus Lactate with Increase of Pyruvate Due to Presence of Methylene Blue

Experiment No		Increase in loss of glucose + lactate due to presence of methylene blue as mM lactate	Increase of pyruvate due to methylene blue	Difference, mM lactate
			m M	
148	Defibrinated blood	2 4	1.8	0 6
149	" "	2 4	2.0	0 4
126-a	" "	3 9	2.5	1.4
221	" "	2.5	1 1	1.4
IV-2	Erythrocytes + glucose	2.5	1.4	1.1
128	Glycolyzed erythrocytes	2 7	2 1	0.6
150	" "	2 0	1 9	0 1
157-a	Washed erythrocytes	0.9	0.9	0 0
151-b	" " + pyruvate	0.3	0.6	+0.3

TABLE IV

Pyruvic Acid Formation, Oxygen Consumption, and Loss of Oxygen Capacity in Presence of Methylene Blue

Experiment No.		Incubation period	Sugar	Lactic acid	Pyruvic acid	Carbon dioxide	Oxygen Content	Oxygen Capacity
		hrs.	mM per l.	mM per l.	mM per l.	mM per l.	mM per l.	mM per l.
148	Defibrinated blood							
	Initial	0	8.1	2.9	0.1	8.97	11.50	11.50
	Change in control	3	−3.7	+5.5	+0 5			
	" " presence of methylene blue	3	−4.0	+3.7	+2 3	+6.07	−6.88	−2.05
V-34	Defibrinated blood (with added glucose)							
	Initial	0	6.8	2.6	0.2	13.79	10.63	10.88
	Change in control	3	−3.1	+4.6	+0.1	+0.55	−0.68	
	" " presence of methylene blue (0.8 × 10⁻⁴ M)	3	−3.9	+3.9	+1.4	+4.14	−5.66	−0.78
V-35	Defibrinated blood* (with added glucose)							
	Initial	0	9.0	2.9	0.4	17.60	7 88	7.88
	Change in control	3	−3.1	+5.0	0	+2 52	−0 78	
	" " presence of methylene blue (0.8 × 10⁻⁴ M)	3	−3.4	+3.7	+1.5	+3.72	−3 61	−0.18
128	Glycolyzed erythrocytes							
	Initial	0	0.2	10.1	0.0	4.97	11.83	11 83
	Change in control	2¾		+0.9	+0.6	+0.31	−0.39	−0.13
	" " presence of methylene blue	1½		−1.1	+1.6	+0.80	−2 65	−2.23
	Change in presence of methylene blue	3¾		−1.9	+2.8	+0.75	−3 86	−3.70
83-a	Glycolyzed erythrocytes							
	Initial	0	0.2	9.3		4.72	7.72	7.72
	Change in control	2	0	0		+0.99	−0.40	
	" " "	3	0	0		+0.98	−2.92	
	" " presence of methylene blue	2	0	−1.2		+1.32	−2 39	−2.40
	Change in presence of methylene blue	3	0	−1.7		+1.78	−3.67	−3.34
82	Washed erythrocytes							
	Initial	0	0	8.0		2.32	9.42	9.42
	Change in control	3	0	+0.9		+1.38	−0.49	+0.06
	" " presence of methylene blue	3	0	−1.3		+1.03	−2.97	−3.09

' **Rabbit** blood.

sion.   In our first experiments of this sort we were struck by the fact that a loss of oxygen *capacity* always accompanied the oxidation of lactate in the presence of methylene blue.[1]   This loss of oxygen-combining power is explained by the appearance in the solutions, simultaneously with the gradual loss of oxygen capacity, of the absorption band characteristic of methemoglobin.   The bearing of this on the mechanism of the dye catalysis is considered in Paper II.

TABLE V

Summary of Experiment 148 with Defibrinated Blood

	Total carbohydrate as glucose		
	Initial	After incubation (control)	After incubation with methylene blue
	m_M per l	m_M per l.	m_M per l.
Glucose.	8.1	4 4	4 1
Lactate (mM $\times$ 0.5). .	1 45	4.2	3 3
Pyruvate (" $\times$ 0 5)	0 05	0 3	1.2
Total	9 6	8 9	8 6
Decrease in total		0 7	1.0

m_M per l

Total O_2 consumed during 3 hrs. incubation with methylene blue   6 88
O_2 consumed in oxidation of Hb (2.05 mM $\times$ 0.25)   0.51
"   "   "   "   " lactate to pyruvate (2.3 mM $\times$ 0.5)   1.15
"   "   " other oxidations (residual O_2 consumption)   5.22
CO_2 produced = 6.07 mM.   R.Q. (calculated from total CO_2 production and "residual" O_2 consumption) = 1.16

When glucose is present, the oxidation of lactate to pyruvate and of hemoglobin to methemoglobin accounts for only a fraction of the oxygen consumed.   This is illustrated in Table V.

From a summary of this and similar experiments with defibrinated blood and erythrocyte suspensions (Table VI),[2] the following conclusions may be drawn: (*a*) the residual O_2 consumption and CO_2 production are greater in the presence than in the absence of glucose; (*b*) the loss of total carbohydrate in defibrinated blood is

[1] The extent of the loss in O_2 capacity is dependent upon the amount of dye added and is greatly decreased by the presence of glucose (see Paper II).

[2] The data are obtained by one method illustrated in Table V.

greater in the presence than in the absence of methylene blue; (c) the difference between the decrease of total carbohydrate in the presence of methylene blue and that in the control (0.3, 0.6, and 0.4 mM of glucose respectively) is in each of the three experiments with defibrinated blood more than accounted for by the corresponding residual oxygen consumption, assuming complete oxidation to CO_2 and H_2O. Since frequently there is a large and unexplained production of CO_2 in defibrinated blood in the control—a fact pointed out by Harrop and Barron—the respiratory quotients in these experiments are, perhaps, not reliable indices

TABLE VI

Condensed Summary of Experiments in Table IV

The results are expressed in mM per liter.

	Experiment No	Decrease in total carbohydrate expressed as glucose		Residual O_2 consumed in presence of methylene blue	CO_2 produced in presence of methylene blue	R Q in presence of methylene blue*
		Control	Methylene blue			
Defibrinated blood	148	0 7	1 0	5 22	6 07	1 16
(containing glucose	V-34	0 7	1 3	4 76	4.14	0.87
and lactate)	V-35	0 5	0 9	2 81	3 72	1 32
Corpuscle suspensions	128†		0 25	1 54	0 75	0 49
(containing lactate	82			1 55‡	1 03	0 66
but no glucose)	83			1 99‡	1.78	0 90

* Calculated from total CO_2 production and "residual" O_2 consumption.

† Incubated 3.7 hours.

‡ No figures being available on pyruvate formed, these values are arrived at by assuming that the pyruvic acid increase was equivalent to the lactic acid decrease.

to the nature of the substances oxidized. Nevertheless, it appears probable from a limited number of experiments that a considerable part of the glucose not accounted for as lactate and pyruvate is, in the presence of the dye, oxidized to CO_2.

Although the data of Barron and Harrop did not indicate the nature of the substance or substances oxidized, they (2) suggested that, "oxidation may take place in the early stages of dissociation, before the glucose molecule is split into three carbon chain fragments, probably when the glucose molecule is esterified to hexosediphosphoric acid." In a recent paper Barron and Ham-

burger (17) state, "It was also shown in the case of erythrocytes and leucocytes that the increased oxygen consumption was due to the oxidation of lactic acid," citing the paper of Barron and Harrop. We find in the various papers of Barron and Harrop no evidence for the direct oxidation of lactate, which was first demonstrated in one of our reports (3). Furthermore, in the light of our results the statement quoted above must be modified to say that a *portion* of the increased oxygen consumption is due to the oxidation of lactic acid.

Qualitative Identification of Pyruvic Acid—Pyruvic acid was detected in concentrated filtrates from blood after incubation with methylene blue by the Simon (18) nitroprusside reaction[3] and was isolated as the *p*-nitrophenylhydrazone by the procedure described by Hahn, Fischbach, and Haarmann (19). We were not, however, successful in obtaining a quantitative recovery by this method. A loss of 20 to 40 per cent, not preventable by a large excess of semicarbazide, apparently occurs during evaporation. From 200 cc. of corpuscle suspension (containing added lactate) which had been incubated $3\frac{1}{2}$ hours with methylene blue, 55 mg. of a *p*-nitrophenylhydrazone which melted with decomposition at 218-220° (uncorrected) were isolated. (Hyde (20) gives 219–220°, Dakin and Dudley (21) give 223–225° for *p*-nitrophenylhydrazone of pyruvic acid.) The melting point was not altered when the product was mixed with known *p*-nitrophenylhydrazone of pyruvic acid. In dilute sodium hydroxide the material gives the characteristic red color.

Acetaldehyde—In view of the claim by Ray (22) that lactate is oxidized by red cells with formation of acetaldehyde, experiments were carried out to test this possibility. During incubation a current of moist air was passed through the flask containing the control as well as through that containing blood with added methylene blue and thence into absorption towers containing bisulfite. No bisulfite-binding substance was detected by subsequent titration by the Clausen (23) method. In other experiments blood was incubated with $CaSO_3$ (2 gm. of neutral salt to 10 cc. of blood) in

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[3] The sensitiveness of this reaction is increased about 10 times—pyruvic acid being detected at a dilution of 1:50,000— if the pyruvic acid solution is saturated with $(NH_4)_2SO_4$ and stratified rather than mixed with strong ammonia water.

the presence and absence of methylene blue. The results showed that, although $CaSO_3$ has little effect on glycolysis and lactic acid formation in the absence of the dye and does not appreciably affect the disappearance of lactate in the presence of methylene blue, only traces (0.03 to 0.1 mM per liter) of acetaldehyde are formed. The traces probably come from decomposition of protein (Riesser (24)) during the distillation. The amounts were no greater in the presence of methylene blue, or dye plus $CaSO_3$ than in the controls, and we conclude that acetaldehyde is not formed from lactate or pyruvate by dog red blood cells with or without methylene blue. It must be noted, however, that Warburg and Christian (9) have reported that hexosemonophosphate is oxidized by cytolyzed rabbit red blood cells and also by an enzyme system prepared from these with the formation by the latter of acetaldehyde.

SUMMARY

Lactic acid is oxidized to pyruvic acid and hemoglobin to methemoglobin by defibrinated dog blood or erythrocytes in the presence of methylene blue. In the absence of glucose, the amount of pyruvate formed equals or slightly exceeds the decrease of lactate, and is apparently the only product of lactate oxidation by this system. Added pyruvic acid is not attacked. Acetaldehyde was not detected. In the presence of glucose (and lactate, formed by glycolysis) more glucose plus lactate disappears than is accounted for by pyruvate formation. The excess appears to be oxidized not via lactate or pyruvate but by some unknown route to CO_2.

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OXIDATIONS BY ERYTHROCYTES AND THE CATALYTIC INFLUENCE OF METHYLENE BLUE

II. METHEMOGLOBIN AND THE EFFECT OF CYANIDE*

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The marked increase of respiration and the accompanying oxidation of carbohydrate exhibited by mammalian erythrocytes under the catalytic influence of methylene blue (Harrop and Barron (1)) make this semibiological model an instructive one for the study both of carbohydrate oxidation and the catalytic factors essential to respiration. In a preceding paper (2) we reported experiments which show that of the carbohydrate oxidized by this system one part is lactic acid, while another part is some other glucose derivative or, perhaps, the sugar itself. The purpose of the present paper is to present experiments which concern especially the mechanism of the catalysis by methylene blue in this system. Because fairly accurate chemical methods became available for determination of both substrate and product, we chose the less complex oxidation of lactate to pyruvate (in the absence of glucose) as the reaction with which to study the mechanism of the catalytic factors involved.

As explained in Paper I, it was early found (3) that the methemoglobin is formed in the course of the dye catalysis. In several of our first experiments a constant relation—not confirmed in later work—appeared to exist between the amounts of pyruvate and of methemoglobin formed. In attempting to relate these oxidations to the reversible reactions of the dye, we were led astray by this

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* The data are taken from a dissertation presented to the Board of Graduate Studies in partial fulfilment of the requirements for the degree of Doctor of Philosophy, Washington University, June, 1932.

false relation, as well as by misplaced emphasis on the formation of a mol-oxide and H_2O_2 when leucomethylene blue reacts with oxygen, and erroneously supposed methemoglobin to be formed by the action of a leuco dye mol-oxide or by a hemoglobin-leucomethylene blue peroxide complex.   Warburg, Kubowitz, and Christian (4), who also noted the appearance of methemoglobin in red blood cells treated with methylene blue, gave the correct explanation by demonstrating that hemoglobin is directly oxidized to methemoglobin by methylene blue.   Since the leuco dye is oxidized by oxygen, these two reactions accomplish the catalytic oxidation of hemoglobin.   Warburg, Kubowitz, and Christian further showed that methemoglobin, when formed within the cells by other agents, especially amyl nitrite, acts as an oxidant of carbohydrate, the methemoglobin thereby being reduced to hemoglobin.   These authors advanced the view that the action of methylene blue is due *merely* to the formation of methemoglobin and further that methylene blue catalysis of tissue respiration involves in all cases a participation of hemin compounds.

The experiments here reported show that methylene blue may oxidize activated lactate *without* the intermediary participation of methemoglobin.   This evidence concerns the influence of cyanide on the reaction.   In the absence of methylene blue HCN blocks the oxidation of lactate by methemoglobin cells.   *Cyanmethemoglobin is, therefore, not an oxidant of the activated substrate.* In the presence of methylene blue, cyanide inhibition is not only absent, but an excess of cyanide beyond the amount which is bound by methemoglobin actually *accelerates* lactate oxidation.   Semicarbazide also accelerates lactate oxidation.   Both substances appear to exert their accelerating effect by combining with pyruvate and thus removing the oxidation product.   This interpretation would imply a reversibility of the lactate-pyruvate oxidation. In support of this idea we find that increasing the initial concentration of pyruvate definitely retards lactate oxidation.

Methods

Dog erythrocyte suspensions were prepared and incubated in the manner previously described (2).   The concentration of methylene blue unless otherwise stated is 1.3×10^{-4} M.   Methe-

moglobin cells were prepared by treating erythrocytes with 0.9 per cent sodium chloride containing a few drops of amyl nitrite (Warburg, Kubowitz, and Christian (4)).

Solutions of HCN were freshly prepared by bringing NaCN solutions either with H_3PO_4 or HCl to pH 7.4. At this pH the NaCN is 98 per cent converted to HCN, and a 0.15 M solution (as to total cyanide) is isotonic, since only the salt (NaCl or sodium phosphate) is osmotically active. In some instances it was necessary to add hypertonic solutions in order to attain certain high concentrations of HCN. In such cases the controls also were made hypertonic with NaCl or phosphate. The concentration of HCN in the solutions was determined by the method of Kolthoff and Furmann (5). Aqueous solutions of semicarbazide-HCl (m.p. 174°) were adjusted to pH 7.4 for use.

Oxygen capacity was determined in most instances according to Van Slyke and Neill (6). When it was necessary to determine the O_2 capacity of a number of samples of blood simultaneously, the Warburg apparatus was used.

Lactic acid and pyruvic acid were determined as previously described (2) except in those experiments where HCN was present. HCN combines with bisulfite similarly as acetaldehyde, and is liberated by $NaHCO_3$. If not first removed it introduces an error in the lactate results. This fact does not appear to have been generally recognized. Control experiments showed that boiling the acidified filtrate for a few minutes effectively rids the solution of HCN. This preliminary boiling was found to cause an insignificant loss of lactate, amounting to less than 2 per cent. Methylene blue and semicarbazide, in the amounts employed, have no effect on lactic acid determination.

In the presence of HCN pyruvic acid was reduced on the boiling water bath, with the reagents ($NaHSO_4$, Zn, and a very small amount of $CuSO_4$) employed by Kendall and Friedemann (7). Results of analyses of known solutions containing cyanide by this procedure were satisfactory. When cyanide is present reduction at room temperature yields quite low results; presumably the reaction of pyruvic acid with HCN is slowly reversible, the compound being destroyed at boiling temperature with evolution of HCN.

Methemoglobin As Oxidant—Methemoglobin in the absence of cells has, at blood pH, no effect on lactate or glucose. When formed within glycolytically active red blood cells (by amyl nitrite), however, methemoglobin oxidizes glucose and lactate with production of CO_2 and pyruvate and reformation of hemoglobin (Warburg, Kubowitz, and Christian (4)). When methemoglobin oxidizes lactate to pyruvate, 2 molecules of hemoglobin (in terms of O_2 capacity) should be regenerated for each molecule of lactate oxidized. This relation should hold in the presence or absence of oxygen. If other substances are simultaneously oxidized by methemoglobin a higher ratio of oxygen capacity increase to lactate oxidized should result. To test this relation, washed methemoglobin cells were incubated in an isotonic buffer solution containing lactate both aerobically (in large flasks containing air) and anaerobically (in filled, sealed tubes). At intervals portions were analyzed for lactate, pyruvate, and oxygen capacity. A series of separate tubes was used for the anaerobic samples. The principle results of a representative experiment are shown in Fig. 1.

The rate of pyruvate formation is the same in the presence and in the absence of oxygen, and is much greater than corresponds to the methemoglobin reduced. For example, after 3 hours the pyruvate had increased 1.5 mM above the initial value. This would require the reduction of 3 mM of methemoglobin, with an equivalent increase of oxygen capacity. With oxygen excluded during incubation the oxygen capacity increased only 1.2 mM, or 40 per cent of that required if methemoglobin were the only oxidant. With oxygen present during incubation a larger amount of hemoglobin, 1.8 mM, was formed, but this represents only 60 per cent of the oxidation equivalents necessary to account for the pyruvate. The discrepancy appears to mean either that some other oxidant besides methemoglobin is created by the nitrite treatment and that this also oxidizes lactate, or that, as suggested by Hartridge (8) as well as by Warburg, Kubowitz, and Christian (4), nitrite "methemoglobin" is not ordinary methemoglobin. Our experiments suggest that it may be a substance having a higher oxidizing equivalence.

Michaelis and Salomon (9) report that some hematin as well as

methemoglobin is formed by the action of amyl nitrite on hemoglobin. To the extent that this occurs in the cells or that methemoglobin is otherwise denatured, the reduction would not be accompanied by a rise of oxygen capacity. The explanation is not now clear.
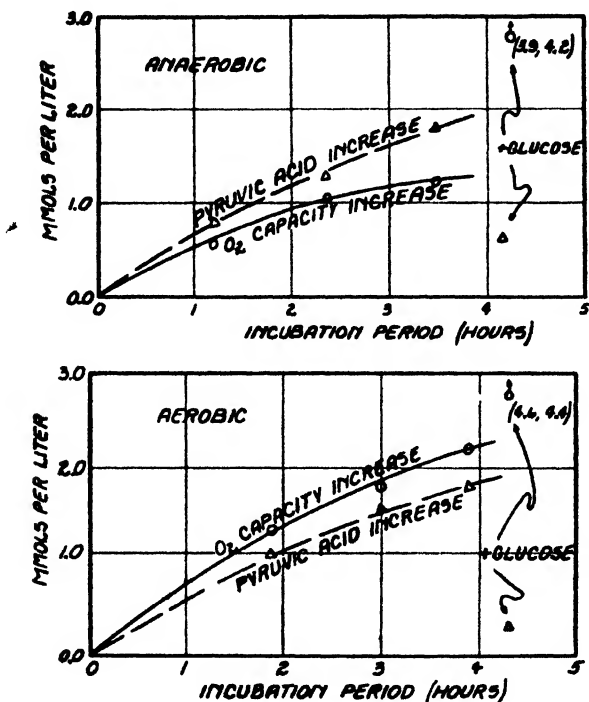
Fig. 1. Oxidation of lactic acid by methemoglobin erythrocytes. Effect of glucose.

The fact that the presence of oxygen during incubation favors the reduction of methemoglobin may perhaps be due to the removal of hemoglobin as formed (by its fixation as oxyhemoglobin) thereby accentuating the participation of methemoglobin as an oxidant and sparing the other oxidant. Points are shown on Fig. 1 to represent results when glucose as well as lactate is present. Glucose greatly accelerates the rate of methemoglobin reduction, both anaerobically and aerobically, while the pyruvate formation

is at the same time much less. This indicates a preferential oxidation of glucose over lactate by nitrite-methemoglobin cells.

It has already been noted that a solution of methemoglobin has no action on a solution of sodium lactate; *activation of the substrate is necessary.* The following experiment was performed to ascertain whether methemoglobin in solution *outside* the cell would accomplish lactate oxidation. Normal dog erythrocytes were suspended in an equal volume of an isotonic NaCl-phosphate solution (pH 7.4) containing lactate and methemoglobin. The final concentration of the latter by spectrophotometric measurement was about 6 per cent. The suspension was incubated $3\frac{1}{2}$ hours at 37.5°. The data in Table I show a small increase of

TABLE I

Normal Erythrocytes with Methemoglobin in External Solution (Incubation $3\frac{1}{2}$ Hours at 37°)

The results are expressed in mM per liter.

	Lactic acid		Pyruvic acid	
	Initial	After incubation	Initial	After incubation
Cells in MHb solution............	6 0	6.5	0 0	0.5
Same + 200 mg. per cent glucose.....		10.6		0.5
" + methylene blue.........		4 7		2.3

pyruvic acid by the cells incubated with external methemoglobin, but very much less than the amount formed in the presence of methylene blue. The cells were glycolytically active, as is shown by the increase of lactate from added glucose. The lactic acid activation appears to take place *within* the cell rather than at the outer surface. The immediate oxidant, therefore, must be inside the cell.

Effect of Cyanide on Lactate Oxidation by Methylene Blue— Early in our experiments the effect of cyanide on the oxidation of lactate by red blood cells and methylene blue was tested. Moderate concentrations of HCN, up to about 0.01 M, were found to exert a slight inhibition on the rate of lactate disappearance. Higher concentrations, about 0.015 M and above, caused an *increase* in the rate of lactate loss. These observations acquired especial

interest when Warburg, Kubowitz, and Christian (4) claimed that methemoglobin, and not methylene blue, is the immediate oxidant of the organic substrate. Table II shows a summary of three experiments in which washed dog erythrocytes were incu-

TABLE II

Effect of HCN on Methylene Blue Catalysis of Lactate Oxidation by Dog Erythrocytes (Initial Hemoglobin 0.01 м, Incubation 3 Hours at 37°)

The results are expressed in mм per liter.

	HCN concentration	Without methylene blue		With methylene blue (1.4 × 10⁻⁴ м)	
		Lactate	Pyruvate	Lactate	Pyruvate
	м				
Experiment A	Before incubation	7 8	0 0	7 8	0.0
	Change after incubation				
	0	+0 9	+0 1	−0 9	+1 8
	0 02	+0 4	+0 5	−2 6	+3 4
	0 10	−0.5	+1.1		
Experiment B	Before incubation	11 4	0 2	11 4	0 2
	Change after incubation				
	0			−1 6	+2.3
	0 005	+1 1	+0 1	−1 7	+2.6
	0 010	+1 2	0 0	−1 6	+1.9
	0 015	+0 9	0.0	−2.8	+2.9
	0 020	+0 9	+0 1	−4.0	+4 1
Experiment C	Before incubation	10 9	0 0	10 9	0 0
	Change after incubation				
	0			−1 2	+1 9
	0 01	+0 4	0 0	−1 3	+1.3
	0 02	+0 2	+0 5	−3 3	+3.5
	0 02 (Heated)			+0.3	0.0
	0 04	+0 1	+0.6	−4 3	+4.5
	0.06	+0.2	+0 5	−4.6	+5.1
	0 08	−0 2	+1 0	−4.7	+5 0
	0 10	−0 2	+1.0	−4 5	+4 9

bated with lactate in the presence of different concentrations of HCN with and without methylene blue. To simplify presentation, the results are given as *changes* in lactate and pyruvate which took place during incubation. The initial values, from which the total amounts present in each case may be obtained, are also given.

In the absence of methylene blue and HCN the red blood cells do not attack lactate, and HCN up to about 0.015 M has little effect on lactate oxidation by the dye.   The slight increase of lactate in the controls probably originates from hexosephosphate or from small residues of glucose not removed in the preliminary washing. With higher concentrations of HCN some pyruvate is formed even without the dye.   This may be due in part to decomposition of hemoglobin and formation of some cyanhematin.   The rate at which cyanide causes decomposition (loss of oxygen capacity) of buffered oxyhemoglobin solutions (pH 7.4) is greatly accelerated by the addition of a small amount of hematin.   Presumably cyanhematin shows this property through catalytic reduction and reoxidation.   When formed within the red blood cell by high cyanide concentration, cyanhematin may perhaps similarly catalyze the oxidation of lactate.   If this is the correct explanation, cyanhematin behaves differently from cyanmethemoglobin.   In the presence of methylene blue cyanide exerts no inhibition on the dye catalysis when the equivalent HCN concentration is below that of the hemoglobin—in these experiments, 0.01 M.   With 0.01 M HCN the oxidation is partially inhibited if judged by pyruvate formation, but not according to lactate disappearance.   With increasing concentrations of HCN above this value the amount of lactate oxidized rises progressively up to 0.06 M cyanide, beyond which no further effect is noted.[1]   Heating the blood cell suspension to 90° before adding dye and HCN destroyed the power of oxidizing lactate.

Fig. 2 shows graphically the extent to which 0.02 M HCN increases the rate of lactate oxidation and also the rate of loss of oxygen capacity (methemoglobin formation) of dog erythrocytes under the influence of methylene blue.   The two broken lines represent the rates of lactate oxidation.   The two solid lines represent the rates of loss of oxygen capacity.   The former is approximately doubled and the latter is increased about 8 or 10 times by 0.02 M HCN.   The increased rate of hemoglobin oxidation is doubtless due, as Warburg states, to the formation of the slightly dissociated cyanmethemoglobin.

[1] Recent experiments indicate that the higher concentrations of HCN are effective in further increasing lactate oxidation if the initial lactate concentration is increased.   This suggests that only one of the isomeric forms of lactic acid is attacked.

The following experiment shows that cells containing an abundance of cyanmethemoglobin and the usual amount of methylene blue do not oxidize lactate except in the presence of oxygen. 50 cc. of washed dog erythrocytes were suspended in 25 cc. of a medium composed of lactate, NaCl, HCN, and methylene blue. After incubating in air 50 minutes, at which time all of the hemoglobin had been oxidized, a sample was removed for analysis and the remainder was incubated, one part in a flask (aerobic) and the
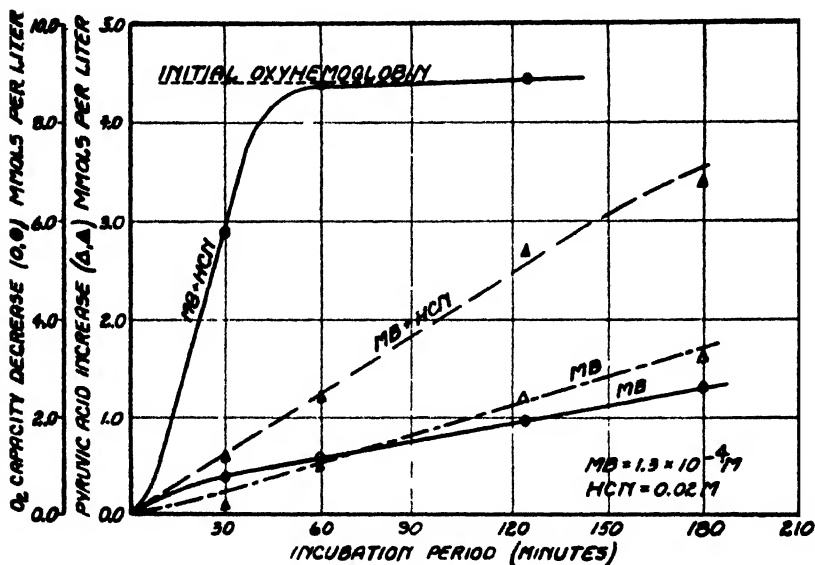
Fig. 2. Effect of HCN on rates of lactic acid and hemoglobin oxidation. MB = methylene blue.

other in a closed filled tube (anaerobic) an additional 165 minutes. The results are shown in Fig. 3. The slight increase of pyruvic acid in the anaerobic tube can be accounted for by the dissolved oxygen and methylene blue.

Effect of Cyanide on Lactate Oxidation by Methemoglobin Cells— The results in Table III show that 0.02 M cyanide completely prevents the oxidation of lactate by methemoglobin cells. The addition of methylene blue alone to methemoglobin cells slightly increases the oxidation, being virtually equivalent to the addition
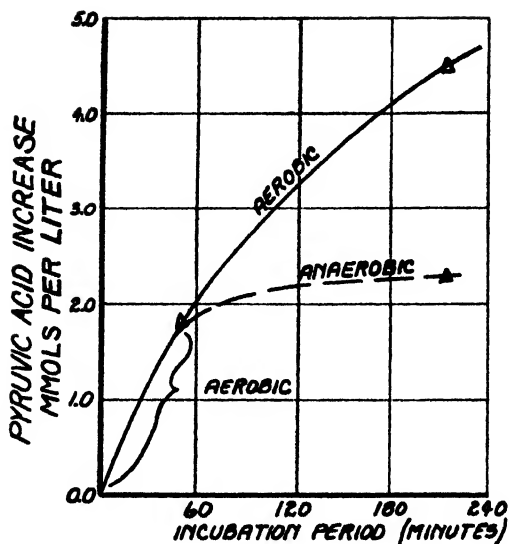
Fig. 3. Inhibition of accelerating action of HCN by anaerobiosis. Cells = 2 volumes. Medium = 1 volume. Methylene blue = 1.7×10^{-4} M. HCN = 0.026 M.

TABLE III

Inhibition by HCN of Lactate Oxidation by Methemoglobin (Amyl Nitrite) Cells

The results are expressed in mM per liter.

		Lactic acid	Pyruvic acid
Experiment A	Initial	10 5	0.0
	Change after incubation		
	MHb cells	−1.4	+1.6
	Same + 0.02 M HCN	0.0	0.0
	" + methylene blue	−1.7	+2.1
	" + HCN + methylene blue	−3.8	+4 0
Experiment B	Initial	10.6	0.3
	Change after incubation		
	MHb cells	−1.1	+1.1
	Same + 0.025 M HCN	−0.1	−0.3
	" + methylene blue	−1.3	+1.7
	" + " " + HCN	−4.0	+3.1

of methylene blue to normal cells. The addition of methylene blue sets aside the cyanide inhibition. Or stated the other way, cyanide inhibits the oxidation by methemoglobin but not that by methylene blue.

Relation of Cyanide Acceleration to Methylene Blue Concentration—As a means of deciding whether the cyanide acceleration of lactate oxidation is concerned with oxygen activation or with other factors, such as substrate activation, experiments were based on the following argument. Within a certain low range of concentrations
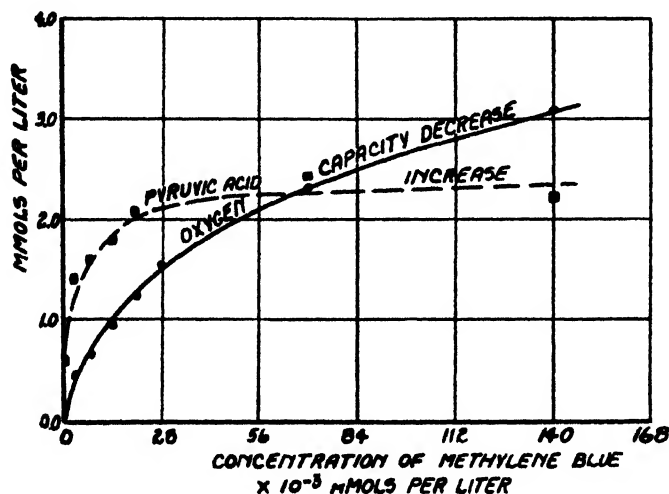
Fig. 4. Effect of methylene blue concentration on amounts of lactic acid and hemoglobin oxidized (incubated 3¼ hours at 37.4°).

of methylene blue (no cyanide being present), the lactate oxidation by erythrocytes rises progressively with increasing dye concentrations, while above this range increasing the dye concentration has much less effect. Fig. 4 illustrates this statement, and demonstrates also the effect of dye concentration on the rate of hemoglobin oxidation. The latter continues to increase with increasing concentration of dye. In the low range the rate of lactate oxidation is apparently *limited by the dye concentration*, while above this range, where the rate of lactate oxidation is almost independent of dye concentration, *substrate activation* must be the

limiting factor.   If cyanide acceleration is concerned with sub-
strate activation, it should be less evident when the dye concen-
tration is the limiting factor than when the dye is present in excess.
Only in the last case can an increase of substrate activation result

TABLE IV

*Relation of Cyanide Acceleration to Methylene Blue Concentration.   Washed
Dog Erythrocytes (Incubation with Lactate 3 Hours at 37°)*

The results are expressed in mM per liter.

		Lac-tate	Pyru-vate	Oxygen capacity
Experiment A	Control without methylene blue or HCN			
	Before incubation	9 4	0.3	
	After "	10 2	0 8	
	Difference from incubated control			
	HCN (0.05 M)	−0 6	+0 5	
	Methylene blue (1 4 × 10^{-6} M)	−0.9	+0 7	
	" " (1 4 × 10^{-6} ") + HCN (0.05 M)	−1.5	+1 3	
	Methylene blue (1 4 × 10^{-4} ")	−2.3	+1.7	
	" " (1.4 × 10^{-4} ") + HCN (0.05 M)	−6.2	+5.2	
Experiment B	Control without methylene blue or cyanide			
	Before incubation	10.8	0 6	9.96
	After "	11.6	0.7	9.24
	Difference from incubated control			
	HCN (0.02 M)	0.0	0 0	−1.96
	Methylene blue (1 4 × 10^{-6} M)	−0.9	+0.8	−0.4
	" " (1.4 × 10^{-6} ") + HCN (0.02 M)	−1.2	+1 3	−6 07
	Methylene blue (1 4 × 10^{-4} ")	−1 8	+1.6	−2 9
	" " (1.4 × 10^{-4} ") + HCN (0.02 M)	−4.1	+3 7	−9 2

in increased oxidation.   The question appears to be answered by
the results in Table IV.

With only 1.4 × 10^{-6} M methylene blue the amount of lactate
oxidized (in excess of the incubated control) in the presence of both
dye and 0.05 M HCN is the same in Experiment A as the sum
of the two separately; that is, there is no catalytic effect of the

cyanide on the oxidation by this concentration of dye. In Experiment B, with 0.02 M HCN, the effect of cyanide at the low dye concentration is distinct, but small as compared with the large effect of cyanide with 1.4×10^{-4} M methylene blue. According to this evidence, the accelerating effect of cyanide on lactate oxidation is not concerned with oxygen activation or transport but is probably related to substrate activation.

TABLE V

Effect of Semicarbazide on Lactate Oxidation by Washed Erythrocytes and Methylene Blue

The results are expressed in mM per liter.

	Lactate		Pyruvate	
	Present	Change*	Present	Change*
Before incubation				
Control†	9 5		0.2	
" + semicarbazide 0.025 M	9 4		0 0	
After 3 hrs. incubation				
Control.	10 4		0 4	
With semicarbazide 0.025 M. . .	9 6	−0 8	0 2	−0 2
" methylene blue (1 6 × 10⁻⁴ M) .	8 5	−1 9	2.1	+1 7
" " " + semicarbazide 0.025 M.	6 2	−4.2	2.0	+1 6
" semicarbazide 0.05 M	9 3	−1 1		
" methylene blue + semicarbazide 0.05 M.	5 7	−4.7	1.7	+1 3
" " " + HCN 0.05 M.	4 8	−5.6		

* Change from incubated control.

† Control contained added phenol red used as indicator in preparing semicarbazide and HCN solutions. This dye has no effect on the oxidations.

Effect of Semicarbazide—The fact that HCN combines with pyruvic acid to form a cyanhydrin suggests the possibility that the accelerating effect on lactate oxidation is concerned with removal of the reaction end-product. If this be the case, other substances which combine with pyruvic acid through the ketonic carbon would be expected to show a similar effect in increasing lactic acid oxidation. Semicarbazide is such a substance, and this also causes acceleration of lactic acid destruction as is shown by the data in Table V. Methylene blue and semicarbazide together, like the

dye plus HCN, cause much greater loss of lactate than separately. Although the missing lactate is not recovered as pyruvate, it seems probable that this is formed. The method of determining pyruvic acid in the presence of semicarbazide gives good recoveries with pyruvate solutions.[2] The lack of good recovery from blood appears to be due to loss of semicarbazone by precipitation with mercuric chloride. According to the lactate results, semicarbazide

TABLE VI

Inhibitory Effect of Initially High Pyruvic Acid on Lactic Acid Oxidation by Methemoglobin Cells

The results are expressed in mM per liter.

		Incubation period	Lactic acid		Pyruvic acid	
		hrs.	Present	Decrease	Present	Increase
Experiment A	Control	0	10 5		0.0	
	Pyruvate added	0	10 5		5.0	
	Control	3	9 1	1.4	1.6	1.6
	Pyruvate added	3	10 4	0 1	5 2	0 2
Experiment B	Control	0	10.6			
	Pyruvate added*	0	10.6			
	Control	3	9 5	1.1		
	Pyruvate added	3	10.3	0.3		
	Methylene blue	3	9 3	1.3		
	" " + pyruvate	3	10.2	0.4		
	0.025 M HCN	3	10.5	0.1		
	Pyruvate + HCN	3	10.4	0.2		
	" + " + methylene blue	3	8.2	2.4		
	Methylene blue + HCN	3	6.6	4.0		

* Concentration = 5 0 mM per liter.

is about as efficient as HCN, and supports the idea that the accelerating effect of HCN is due to fixation of pyruvate.

Effect of High Initial Pyruvic Acid Concentration—If the idea stated above is correct, lactate oxidation should be somewhat re-

[2] The semicarbazone of pyruvic acid is decomposed by strongly acidifying with H_2SO_4 and allowing to stand 24 hours. The pyruvic acid is then determined in the usual way.

tarded by an initially high concentration of pyruvate. The data in Table VI indicate that this is so. Sodium pyruvate (made from freshly distilled acid) as well as lactate was added to the buffer solution in which washed methemoglobin (amyl nitrite) cells were suspended. The presence of 5.0 mM of pyruvate very largely inhibited the lactate oxidation (which occurred in the absence of pyruvate). The added pyruvate was not destroyed. The same inhibition is exerted even when methylene blue is added to the methemoglobin cells. In the presence of methylene blue the inhibition by pyruvate of lactate oxidation is partly removed by HCN, as would be expected from fixation of the pyruvate as cyanhydrin.

<div align="center">DISCUSSION</div>

The rate of oxidation of activated lactate to pyruvate is accelerated by semicarbazide as well as by cyanide, and is slowed by pyruvate. These facts led to the view that this reaction is probably reversible. Its reversibility with enzyme systems has very recently been clearly demonstrated (10). Whether, or to what extent oxidation of lactate to pyruvate occurs in normal intermediary metabolism, therefore, probably depends upon the further disposal of pyruvate.

Cyanide renders methemoglobin inert as an oxidant of activated lactate. Perhaps the chief interest of this fact is that it represents a clear and direct illustration of the view that cyanide inhibition of respiration is due to union of the cyanide with the higher valence form of the "oxygen-transferring enzyme of respiration" (Warburg, Kubowitz, and Christian)—methemoglobin being regarded as analogous to this (4). On the other hand, the fact that when the activity of methemoglobin as an oxidant is abolished by cyanide, the addition of methylene blue leads to resumption of lactate oxidation appears to disprove the view of Warburg, Kubowitz, and Christian (4) to the effect that methylene blue catalysis of respiration in red blood cells depends upon the participation of methemoglobin (Warburg and Christian have since modified their views on this subject (11)).

Whether dye catalysis of respiration is wholly independent of hemin iron cannot be satisfactorily decided. Barron and Hamburger (12) consider this question in a recent paper and conclude

that hemin participation is not essential, because, as they interpret their data, the increased oxygen consumption of marine eggs ("cells deprived of hemoglobin") due to added dyes is "not affected by the addition of increasing concentrations of KCN."   Their results in Table I and Fig. 1 do, however, show a variable but distinct decrease with cyanide.   Also they ignore the uncertainty of cyanide concentrations which results from the rather rapid distillation of HCN from the cell suspensions into the alkali in Warburg vessels (Schmitt and Schmitt (13)).   These points cast some doubt upon the conclusion of Barron and Hamburger. Furthermore, the point should perhaps be left open whether cyanide-insensitive cytochrome or similar hemin compounds may not also be involved in dye catalysis of respiration.

In spite of the fact that a satisfactory general theory of dye catalysis of respiration is at present not possible, the behavior of the methylene blue-erythrocyte system as regards lactate oxidation suggests the following concept.

In systems containing substrate, substrate activator, oxygen, and oxygen activator, the rate of oxygen consumption may be limited either by the rate of substrate activation or by the rate of oxygen activation.   In mammalian erythrocytes the latter is negligible, owing, presumably, to the absence of an oxygen-transferring catalyst.   When this is supplied by adding a suitable dye or by the formation of methemoglobin the system is complete; except in the methemoglobin cell, the oxidant acts only once.   In the artificially completed erythrocyte-lactate-methylene blue system the rate of lactate oxidation is proportional to the amount of dye added, up to a maximum, beyond which more dye has no effect (Fig. 4).   In the rising portion of the curve (concentrations of methylene blue 2×10^{-5} M and less) the rate of oxidation of lactate is limited by the rate of oxygen activation; beyond this concentration of dye, substrate activation becomes the limiting factor, and this is unaffected (unless, perhaps, injured) by the dye.

It may perhaps be inferred from these observations that the widely different effects of added dyes on the respiration of other cells, in some of which respiration is increased markedly and in others not, or even decreased, are to be accounted for in a similar way.   In those cells or systems in which the rate or efficiency of substrate activation exceeds the rate of oxygen activation, dye

catalysis is to be expected; but if the two rates are about equal or if substrate activation is the lower, dyes or other oxygen-activating catalysts should not increase oxygen consumption.

The writer wishes to express his appreciation to Professor Philip A. Shaffer for guidance and criticism in carrying out the experiments reported in this and the preceding paper.

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ELECTROLYTES IN HUMAN TISSUE

I. THE DIGESTION OF TISSUE AND OTHER BIOLOGICAL MATERIAL AND THE SUBSEQUENT DETERMINATION OF VARIOUS ELECTROLYTES[*]

By GLENN E. CULLEN and WALTER E. WILKINS

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Experience with the digestion and analysis of tissue gained during the work on electrolyte content of human tissue reported in Paper II following and in Paper III (14) of this series emphasized the need for an improvement in preparation of tissue and a systematization of the methods used.

In particular, the wet ashing technique, regardless of the catalyst used, is not only exceedingly laborious and unpleasant but is dangerous, both to the operator and to the sample.

To meet this problem we have worked out the system reported here. In general the methods for individual constituents are adapted from standard technique for blood work.

Initial Treatment of Tissue—As soon as possible after removal from the body, the fresh tissue is placed on a clean dry porcelain plate and the fat, tendon, etc., are dissected out as far as practicable. This introduces the greatest error in handling tissue and care must be taken to treat all tissue as uniformly as possible. Care must also be taken not to squeeze out tissue fluid and to reduce evaporation to a minimum. The entire tissue, or portions from different parts of a large sample, is then placed in a weighed Pyrex beaker (of such size that it is not more than one-eighth filled) and

[*] The experimental data in this and the accompanying report are taken from a thesis submitted by Walter E. Wilkins in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biochemistry in the Graduate School of Vanderbilt University.

cut into small pieces of about 1 to 0.5 cc. in size.   This is done with dissecting shears and any expressed tissue juice is carefully wiped back onto the tissue.   The beaker and contents are weighed. The beaker is kept covered with a watch-glass whenever possible.

If chlorides are to be determined, the whole mass is stirred to redistribute all tissue juice uniformly over the tissue, and pieces to make about 1 to 1.5 gm. are taken from different parts of the mixed tissue and placed in a large Pyrex tube for the chloride determination.   The beaker and contents are again weighed. The difference gives the weight for the chloride sample.

Water Content and Dry Weight—The beaker, covered with a watch-glass, is heated in an oven at 105–110° to constant weight. This requires from 10 to 40 hours, depending upon the size of the sample.   The percentage of water is calculated upon the basis of the loss of weight in drying.

Alternative Procedure—In the procedure first used (Paper II), 1 or 2 gm. of tissue are removed, as for chlorides, to a small beaker or weighing bottle and the water content is determined separately. The main bulk of tissue is then dissolved directly in nitric acid without previously drying.

Preparation of Stock Tissue Solution—5 cc. of concentrated nitric acid and 5 cc. of distilled water are added for each gm. of dry tissue, the watch-glass is placed over the beaker, and the mixture is kept in a warm place for about 24 hours.   The mixture is digested slowly on the steam bath until it has become homogeneous except for a small amount of fatty substance which floats upon the surface.   When brown fumes have ceased coming off and the solution is a clear straw-yellow color, after about 2 days, the digest is removed from the steam bath and cooled to room temperature.   It is made up to the smallest convenient volume with distilled water in a volumetric flask, mixed, filtered through an ashless filter paper in order to free it of the fatty substance, and transferred to a Pyrex bottle with ground glass stopper.   Aliquots of this solution are taken for analysis or for preparation of the common ash solution.

Preparation of a Common Ash and Determinations Carried Out on That Ash—Transfer an aliquot of "nitric acid digest" equivalent to about 10 to 15 gm. of fresh tissue to a Pyrex or silica beaker, of such a size that it is not more than one-fourth filled, add 5 to 10 cc.

of 4 N sulfuric acid (or equivalent), and evaporate down to a gummy mass on the steam bath. Add 5 to 10 cc. of freshly prepared ammonium persulfate solution and again evaporate on the steam bath. Evaporate most of the excess sulfuric acid on a hotplate and ash the sample in a muffle furnace overnight at a temperature of 500° or less, and before removing from the furnace raise the temperature to about 600°. Allow the sample to cool in the furnace.

Add 10 to 15 cc. of 1 N HCl and enough water to bring the level of liquid slightly above the level attained by the original sample, bring to dryness on the steam bath, add a few drops of 1 N HCl, dissolve the residue in water, transfer to a 50 cc. volumetric flask with three rinsings of water, and make to volume (a heavy substance, probably containing silicon, may be noted and most of this may be kept in the beaker, or the sample may be filtered through an ashless filter paper after being made to volume). The sample is conveniently stored in a small Erlenmeyer flask. Aliquots of this solution are used for the determination of potassium, sodium, calcium, and magnesium.

Calcium—Calcium determinations are made on aliquots of the ash solution corresponding to 4 to 8 gm. of fresh tissue.

This ash solution is, of course, protein-free and therefore suitable for accurate calcium determinations. As pointed out by Van Slyke and Sendroy (13) the greatest error in calcium determination is in the washing. For the amount of calcium present in tissue digests the ash solution is suitable for any of the various modifications of the oxalate methods for calcium determination.

Proposed New Method for Calcium—In all calcium methods we know of involving the precipitation of calcium oxalate the precipitated calcium oxalate is washed and either the calcium or the oxalate determined. We were working with two methods, the Van Slyke (12) chloride method and the Stadie-Ross (9) sulfate method in which the principle involved was that of measuring the excess of the precipitating reagent. It occurred to us that the same principle could be used of measuring the amount of oxalate in the supernatant fluid after precipitating the calcium with excess oxalate. Many experiments were carried out to determine the accuracy of this procedure.

It was found that with 2 equivalents of oxalate, at pH 5, the

precipitation is complete on standing overnight and that with 4 or more equivalents the precipitation is complete in from $1\frac{1}{2}$ to 2 hours.   On mixtures of pure calcium or protein-free artificial serum or tissue mixtures containing calcium the accuracy of the method equals that of the Van Slyke-Sendroy (13) gasometric method, *i.e.* about 1 per cent.

The new method cannot be used on the supernatant fluid from the Kramer-Tisdall (6) serum technique or on the trichloroacetic acid filtrates of serum because the organic materials present in plasma or trichloroacetic acid filtrates are oxidized to CO_2 by permanganate.

The blank CO_2 formed in the trichloroacetic acid filtrate represents irregularly about 30 per cent of the amount formed in the method.   These solutions must therefore be oxidized.   We have not yet satisfied ourselves as to the optimum conditions for oxidizing this filtrate, but preliminary work indicates that the dry ashing described above with ammonium persulfate and H_2SO_4 is satisfactory.

The method as used for artificial tissue salt mixtures or with solutions of the ash is as follows: An amount of solution equivalent to about 0.2 mg. of calcium is transferred to a volumetric 10 cc. flask, and 1 cc. of 20 per cent sodium acetate, 3 drops of bromcresol green (used here because it eliminates the risk of using alcoholic methyl red solution), and exactly 5 cc. of 0.01 N oxalate solution (diluted each day from 0.1 N) are added.   The solution is adjusted with dilute ammonia water to pH 5 and the volume made exactly to the mark.   The whole is mixed, poured into a 15 cc. centrifuge tube, and capped with a nursing bottle rubber cap. After standing at least 2 hours or overnight the tube is centrifuged about 5 minutes and about 7 cc. of the supernatant liquid transferred with a pipette to a clean tube.   The pipette is manipulated with a rubber tube so that the tip is always well below the surface to avoid any floating oxalate crystals.   The blank for oxalate concentration is made up exactly the same except for the calcium solution.   2 cc. samples are taken for analysis as in the Van Slyke-Sendroy (13) gasometric method.

5 cc. of 0.01 N oxalate represent 5 equivalents of calcium.   If more calcium is present approximately larger amounts of oxalate are required, the relation of calcium and oxalate being always kept

near 1:2 or 1:4.   We prefer using 5 cc. of 0.01 or 0.02 N oxalic acid to measuring 1 cc. of 0.1 N in order to decrease the pipette error. We use oxalic acid so that it may be standardized by titration with a standard alkali.   The calculation is by difference, the Van Slyke-Sendroy (13) tables being used.

Magnesium—Magnesium determinations are made on aliquots of the supernatant fluid from the calcium precipitation, each Ca sample furnishing two aliquots equivalent to 1 to 2 gm. of wet tissue. The Mg is precipitated and washed according to Briggs (2) and determined as $MgNH_4PO_4$ by the Fiske-Subbarow (5) colorimetric method for phosphorus.

Potassium—The ash is suitable for K determinations by either the cobaltinitrite technique or the chloroplatinic acid method. We have had more satisfactory results with the former.   In our experience three requirements must be observed to insure reproducible results with this method.   (1) Each day a portion of the stock cobaltinitrite sufficient for the day is removed from the stock solution, which is kept in the refrigerator, aerated for 15 minutes, and filtered.   (2) With each set of determinations a control is first run on a known K-Na mixture and the factor 0.071 corrected for minor change.   The factor may remain approximately constant for weeks and then suddenly vary greatly.   Usually more vigorous aerating of the cobaltinitrite reagent will correct this.   (3) Temperatures above 25° interfere with the determination.   Therefore, all precipitations and filtrations made during the summer were made in the constant temperature room at 20°.

Two 0.5 cc. portions of the ash solution (usually containing from 0.1 to 0.4 mg. of K) are transferred to 30 cc. beakers, 1 cc. of distilled water added, and 1 cc. of freshly aerated and filtered cobaltinitrite reagent is added drop by drop with continual shaking.   45 minutes are allowed for complete precipitation.

A Shohl (8) filter is prepared by mounting a 1 inch funnel on a suction flask, a glass bead dropped into the funnel, and a mat of asbestos prepared by pouring 3 or 4 cc. of a 1 per cent asbestos suspension on the bead.   The asbestos is pressed into a smooth *firm* mat by means of a wooden applicator.

The precipitated potassium cobaltinitrite with its supernatant fluid is poured onto this filter and is washed about 5 times from the beaker with about 4 cc. of distilled water each time.   The wash

water may be sprayed into the beaker each time from a small wash bottle. The sides of the beaker should be thoroughly washed while being rubbed with a glass rod.

The filter and bead are pushed into the beaker by means of a wooden applicator introduced into the bottom of the funnel, and the funnel is rinsed with about 1 cc. of water. Care should be taken to see that none of the precipitate remains in the funnel. About 3 to 5 cc. of 0.01 N $KMnO_4$ (depending upon the size of the sample) are added from a 10 cc. microburette, followed by 1 cc. of 4 N H_2SO_4. The beaker is inserted into a wire triangle and heated on a boiling water bath for $1\frac{1}{2}$ to 2 minutes (during this time it is removed, the contents mixed by rotating the beaker, and replaced twice). Care is taken that none of the yellow precipitate remains out of contact with the main body of the mixture and that all of it is dissolved before the oxalate is added. After heating, the beaker is removed from the bath and exactly 2 cc. of 0.01 N sodium oxalate are added. The mixture is titrated to the first definite pink from the same burette. A blank of 0.05 cc. is subtracted for end-point color. The final titration should be carried out as rapidly as is consistent with accuracy in order that the end-point may be reached while the solution is still hot.

The addition of the initial $KMnO_4$ for oxidation as well as for titration from the same burette is not only convenient but reduces the error. When it is evident from the rate of decolorization of the $KMnO_4$ that not enough has been added, more can be added without loss of the determination. Further, the total volume delivered from the burette is the quantity used in the determination.

Sodium—The sodium determinations are made according to the technique of Barber and Kolthoff (1), the phosphate being removed by the method of Butler and Tuthill (4).

About 25 cc. of the original ash solution (containing 5 to 8 gm. of wet tissue) are poured into a 50 cc. Erlenmeyer flask, about 0.5 gm. of $Ca(OH)_2$ and a small drop of phenolphthalein solution are added, the flask is stoppered, and the contents are well shaken. The pink color develops and then fades. The flask is allowed to stand for 45 to 60 minutes with occasional shaking. The mixture is then filtered through a No. 40 Whatman ashless filter paper. One 20 cc. portion or two 10 cc. portions of the filtrate are trans-

ferred to 30 cc. beakers and are brought to dryness on the steam bath. 1 cc. of water is added, followed by 1 to 3 drops of concentrated HCl to dissolve partly the $CaCO_3$. Then 15 to 20 cc. of sodium reagent are added and the mixture is well stirred with a small Pyrex rod which is left in the beaker.

1 hour is allowed for complete precipitation. The mixture is filtered through a weighed Jena crucible (fine, No. 4) with suction, two or more 5 cc. portions of sodium reagent being used to rinse the beaker. The precipitate is then washed with five 2 cc. portions of 95 per cent alcohol (saturated with the salt which is being washed and freshly filtered) and finally with two or three 5 cc. portions of ether. Air is drawn through for about 2 minutes after the ether has gone through. The crucible is then removed, wiped dry on the outside and around the inside of the bottom, and is weighed after 10 to 20 minutes.

The control sodium determinations made on an artificial tissue digest gave results consistently about 3 per cent below theoretical.

Total Base—In Paper II of this series phosphorus was removed from the wet ashed sample, equivalent to 1 to 2 gm. of fresh tissue, in a 50 cc. volumetric flask by the Stadie-Ross (9) method, without any apparent loss of sodium or potassium. But in preliminary determinations in the later studies (Paper III (14)) in which 10 to 15 gm. of tissue were ashed, it was found that in a 100 cc. volumetric flask variable amounts of all four bases were lost in the removal of phosphate by the Stadie-Ross method. This was particularly true of calcium and magnesium. For this reason the method was abandoned. Brown and Shohl (3) have since called attention to this fact, and Wright and Allison (15) have recently given a procedure by which they state that this loss may be avoided.

Analyses Made on Nitric Acid Digest. Phosphates—Since phosphate may be lost during ashing in the muffle furnace, it is determined on an aliquot of the nitric acid digest.

Duplicate aliquots of the nitric acid digest containing 0.5 to 1.0 gm. of tissue (wet weight) are transferred to 50 cc. sillimanite crucibles, 1 cc. of 30 per cent $Mg(NO_3)_2$ solution and about 0.1 gm. of MgO are added to each, and the mixtures are stirred by means of a small stream of distilled water from a wash bottle. They are brought to dryness on the steam bath, placed in pipe-stem triangles, and heated *gently* (in hood) with a low flame of a Bunsen burner for

20 to 40 minutes; they are then heated over a full flame for 2 to 5 minutes.   A spongy *white* ash should be obtained.   After the crucibles have cooled, the ash is thoroughly wet with water, about 15 cc. of 2 N H_2SO_4 are added, and the crucibles are heated on the steam bath until the ash has completely dissolved.   If the ash does not dissolve within a few minutes, additional 2 N H_2SO_4 may be added, but a great excess of acid should be avoided.   The solutions are made up to 50 cc. in a volumetric flask and are transferred to 50 cc. Erlenmeyer flasks and stoppered.

Duplicate aliquots, equivalent to 0.1 to 0.2 gm. of wet tissue are transferred to 50 cc. volumetric flasks and the phosphate determined by the Fiske-Subbarow method.   Blanks must be run for the $Mg(NO_3)_2$ and MgO added.

Calcium and Magnesium Determinations Made Directly on Nitric Acid Digest—Before the general ashing technique given above had been worked out and because of the danger of losing calcium and magnesium in the ferric hydroxide method for the removal of phosphates, a great many experiments were made to ascertain whether calcium and magnesium could be accurately determined on the nitric acid digest without ashing.   It was found that if large samples, representing 2 to 6 gm. of tissue, were used, the following technique proved to be accurate within 5 per cent.   It is presented here since the results in Paper II of this series are based upon it and because it is convenient if only calcium and magnesium are to be determined in tissue or if a muffle furnace is not available.

Duplicate aliquots of the digest were brought almost to dryness in small beakers on the steam bath, allowed to cool, 1 drop of nitric acid and 2 cc. of water added, and the mixtures well stirred.   They were transferred to 15 cc. Pyrex centrifuge tubes, the beakers rinsed twice with 1 or 2 cc. of water, once with 1 to 2 cc. of 2 per cent ammonia to dissolve the last traces of protein, and finally with 1 to 2 cc. of water, with care in each instance that the walls of the beaker were well rinsed.   From this state the technique is the same as that outlined above.

Analysis of Tissue.   Chlorides—Van Slyke's (12) microtechnique was used.   Since this must be done on wet tissue (Sunderman and Williams (11)), 1 to 1.5 gm. of fresh tissue are placed in a 100 cc. Pyrex tube with 3 cc. of $AgNO_3$-NHO_3 reagent, the tube is stoppered loosely with a closed end funnel, and the sample

digested *in* a steam bath overnight.   The excess silver is titrated with 0.02 N KCNS with ferric alum as indicator.

Because relatively few reports of this technique are available Table I is given to illustrate the reproducibility of the method.[1]

Reagents—The details of the reagents used in this system are available in the book by Peters and Van Slyke (7), or in the literature cited.

Apparatus—During the early part of the work reported in Papers II and III (14), it was felt necessary to use silica dishes. The ashing technique was all worked out in platinum.   It has been found that Pyrex (general ashing) and sillimanite (ashing for P) are entirely satisfactory.

TABLE I

Chloride Determination on Duplicate Portions of Four Tissues from One Individual (J.C.)

Tissue	Weight of sample of fresh tissue	Chloride found per 100 gm of fresh tissue
	gm.	*mg.*
Gastrocnemius	0 934	226
	1.110	224
Left ventricle	1 350	136
	0 875	135
Liver	1 574	169
	1 430	168
Kidney	1 545	253
	1 090	253

The heating units of the muffle furnace were rearranged by wiring them to a standard electric stove three position switch.   One position on our furnace corresponds to about 500°.   A pyrometer is essential to uniform technique.   A Brown instrument was used in this work.   We feel strongly that all ashing operations in muffle furnaces should be controlled by accurate temperature readings. Description of heating to various "degrees of redness" is practically impossible of accurate duplication in other laboratories.

[1] As this paper goes to press, Sunderman reports (Sunderman, F. W., *J. Biol. Chem.*, **100**, xci (1933); *Proc. Am. Soc. Biol. Chem.*, **8**, xci (1933)) that the open Carius method of Van Slyke does not give complete recovery of tissue chloride but that a preliminary alkaline digestion is necessary.

Stadie and Wright (10) have recently reported an inexpensive pyrometer for this purpose.

When the ashing involves volatilization of large amounts of salts which may sublime near the door or of acids which corrode the heating units of the furnace it is convenient to insert a small Pyrex tube connected to a suction pump in a groove made for the purpose at the edge of the door.   This allows the drawing of a current of air across the beaker from the opening for the thermocouple and aids greatly in removing undesirable fumes.

DISCUSSION

The method for tissue analysis outlined above is based on the preparation of a solution and preliminary partial digestion with nitric acid.   It requires a minimum of working time and labor. The digestion and partial oxidation to a clear solution allow all determinations to be made on uniform aliquots of the sample.   A possible error is the separation of a small amount of fat-like globules which may contain a trace of P.   Repeated ashing tests showed no trace of residue after ignition.

A great number of experiments have been made in working out the optimum conditions for each substance.   Economy of space prohibits publishing these figures but they can be summarized by the statement that the average accuracy obtained is at least that found for the corresponding analyses of blood.

SUMMARY

A system is outlined for the analysis of inorganic substances in tissue based upon a preliminary solution and partial digestion of the tissue with nitric acid followed by ashing in a muffle furnace.

Modifications of existing methods for the determination of phosphorus, calcium, and magnesium are described.

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ELECTROLYTES IN HUMAN TISSUE

II. THE ELECTROLYTE CONTENT OF HEARTS AND OTHER TISSUES FROM CASES WITH VARIOUS DISEASES*

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In recent years a great amount of information has been amassed concerning the influence of various pathological conditions upon the electrolyte distribution in blood. It has become increasingly clear that, important as this information is in itself, it is of greater value in reflecting the changes in the tissue cells. It is desirable to obtain more knowledge of the electrolyte condition in the tissues themselves.

In the present report are presented data from nineteen autopsies on human subjects (Table I) including determinations of chloride, potassium, total phosphorus, and total base in skeletal muscle (gastrocnemius), heart muscle, and in liver and kidney. Magnesium and calcium also were determined in the muscle tissue.

These nineteen patients died from a variety of diseases, in most cases with several complications, and it therefore does not appear justifiable as yet, to attempt to classify these findings with specific diseases. A comparison of the electrolyte concentration of normal and diseased heart muscle is reported elsewhere (Wilkins and Cullen (18)).

Methods

The initial treatment of tissue for the results reported here is the alternative method outlined in the preceding paper (Cullen and Wilkins (4)); *i.e.*, the water content was determined on a 1 to 2 gm. sample of tissue and the fresh tissue was dissolved in nitric acid.

TABLE I

Individual Case Data

Subject	Sex	Age	Weight of heart	Chief diagnosis
		yrs.	*gm.*	
1	M.	36		Pericarditis
2	"	57	430	Carcinoma of esophagus
3	"	40	300	Tuberculosis, adhesive pericarditis
4	"	25	300	Glioma cerebri
5	F.	19	200	Fracture of skull, extradural hemorrhage
6	M.	40	510	Syphilitic aortic insufficiency, myocardial hypertrophy and dilatation
7	F.	38	180	Lymphosarcoma
8	"	64	200	Brown atrophy of heart
9	M.	73	650	Myocardial hypertrophy and dilatation, generalized arteriosclerosis
11	"	65	560	Acute tubular nephritis, syphilitic aortic insufficiency, myocardial hypertrophy
12	"	50	600	Cardiac hypertrophy and dilatation
13	"	61	310	Carcinoma of gallbladder
14	"	38	610	Coronary arteriosclerosis, myocardial hypertrophy and dilatation
15	"	57	450	Cerebrospinal meningitis, hypertrophy of left ventricle
16	F.	42	210	Pulmonary tuberculosis
17	M.	57	270	Medullary carcinoma of stomach
18	"	26	400	Pneumococcus septicemia, pericarditis
19	"	42	510	Syphilitic aortic insufficiency, myocardial hypertrophy and dilatation

Duplicate or triplicate aliquots containing 1 to 2 gm. of tissue were then wet ashed with the aid of superoxol as described by Stadie and Ross (15). A fume trap similar to that used by Folin for micro-Kjeldahl determinations was used to prevent loss from spattering. The digest was transferred to silica beakers, the excess acid driven off, and the whole made to 10 cc.

TABLE II

Inorganic Constituents of Muscle Based on 100 Gm. of Wet Tissue

Case No.	Right ventricle							Left ventricle							Gastrocnemius						
	H₂O	Cl	P	K	Ca	Mg	Total base	H₂O	Cl	P	K	Ca	Mg	Total base	H₂O	Cl	P	K	Ca	Mg	Total base
	gm.	mg.	mg.	mg.	mg.	mg.	m.-eq. per kg.	gm.	mg.	mg.	mg.	mg.	mg.	m.-eq. per kg.	gm.	mg.	mg.	mg.	mg.	mg.	m.-eq. per kg.
1	79.0	95	135	229	3.7	20.3	135	80.1	94					108	78.1	80	182	350	10.6	21.9	139
2	80.2		127	171	7.1	15.5	123	76.2		165	306		22.0	104	76.3	65	155	314	5.2	21.1	124
3	80.2		158	267	6.7	19.1	108	78.2		177	225	7.6	19.9	111	80.5		145	280	5.8	17.9	113
4	80.2		137	229	5.6	17.5	106	80.3		193	345	6.1	23.7	121							
5	76.4						96	80.3		165	290	3.7	22.9								
6	81.3	119	128	142	6.9	10.5		79.6	99	150	162	7.0	19.8	104	80.2	72	111	142	6.1	14.1	67
7	81.4		130	214			108	77.6	82	201	255	5.7	22.3	117	77.8	95	134	190	10.5	12.4	111
8	82.6	204	121	197	11.8	23.5	145	79.5	178	141	265	7.2	23.9	146	81.6	158	161	147	12.7	21.4	142
9	79.7	130	180	284	7.0	21.9	135	78.0	138	201	320	7.8	21.2	145	69.8	210	169	307			141
10	81.4	147	145	248	8.6	17.5	141	81.6	129	156	278	6.8	11.8	146	73.0	68	176	330	6.0	16.8	144
11								78.0	136	143	322			140	81.4	225	113	265			165
12	82.4	157	125	193	7.1	16.5	110	78.7	126	188	231	5.2	19.6	123	70.3	112	156	248	5.4	17.3	115
13	79.6	130	139	241	10.4	24.9	146	79.4	124	160	364	8.0	24.7	190	76.4	66	187	114	9.4	16.0	164
14	82.5	167			6.7	14.7		81.6	160			5.1	15.9		82.4	122	124	236	5.3	16.9	117
15	79.7	90	166	248	5.6	21.9	110	79.4	103	185	301	4.1	26.5	126	71.8	39	198	316	4.0	23.7	122
16		92	154	220	8.8	18.5	125	78.8	87	172	245	4.1	19.5	115							
17	80.9	174	116	219	6.7	16.3	177	80.9	125	161	233	8.4	18.0	134	79.2	100	113	212	11.2	19.6	101
18	77.6	116	144	195	5.9	18.8	105	80.9	92	204	316	9.4	24.8	117	77.7	84	150	264	3.6	22.3	129
19	80.4	163	143	192	8.0	17.5	135	80.5	128	165	256	9.6	22.2	138	82.1	172	122	218	7.9	16.4	145
Average	80.3	137	141	218	7.3	18.4	125	79.3	120	173	277	6.4	21.1	129	77.4	111	150	246	7.4	18.4	127

The phosphates were removed and the total bases determined by the technique of Stadie and Ross.   Potassium was determined after removal of phosphate by the cobaltinitrite method with the precaution outlined in Paper I.   Two or three potassium determinations were made on each of the duplicate or triplicate ashings of each tissue.   Magnesium and calcium were determined directly on the nitric acid digest as described in Paper I.

TABLE III

Inorganic Constituents of Liver and Kidney Based on 100 Gm. of Wet Tissue

Case No.	Liver					Kidney				
	H_2O	Cl	P	K	Total base	H_2O	Cl	P	K	Total base
	gm.	*mg.*	*mg.*	*mg.*	*m.-eq. per kg.*	*gm.*	*mg.*	*mg.*	*mg.*	*m -eq per kg.*
1	78.3	219	184	165	140	81 5	206	188	175	181
2	79.5	119				82.1	159			
7	79.7	107	248	238	114	83 4	97	166	210	129
8	77 5	165	305	235	127	87 4	252	127	97	121
9	75.0	175	304	320	129	81 3	194	158	235	135
10	76.9	157	302	266	148	83 9	240	179	190	144
11	74 9	169	221	326	165	83 3	253	192	169	200
12	78 1	141				82.5	185			
13	76 9	152	195	221	94	76 0	198	166	94	74
14	77.0	160				86 7				
15	75.4	89				82.2	152			
17	77.7	156				84.7	205			
18	78 2	94				85 4	177			
19	74 8	191	167	153	137	83 4	181	164	202	153
Average	77.1	150	241	240	132	83.1	192	168	172	140

Results

The values for the concentrations for the several elements, expressed in terms of wet tissue, are given for the three muscles in Table II and for liver and kidney in Table III.   Since previous work has often been given in terms of dry weight and since it is desired to compare the osmotic concentration of the electrolytes, a summary of the average, maximum, and minimum values for each electrolyte, calculated to dry weight and to milli-equivalent per kilo of tissue water is given in Table IV.   Phosphorus is cal-

culated on the basis that 1 mM of phosphorus binds 1 mM of base (Peters and Van Slyke (10), p. 1102).

Water—Water comprises so large a part of the soft tissues that, even when the absolute amount is markedly increased or decreased,

TABLE IV

Inorganic Constituents Calculated to Dry Weight of Tissue and to Milli-Equivalents per Kilo of Tissue Water

		Mg per 100 gm. dry tissue					M-eq per kilo tissue H₂O				
		Right ventricle	Left ventricle	Gastrocnemius	Liver	Kidney	Right ventricle	Left ventricle	Gastrocnemius	Liver	Kidney
Chloride	Maximum	1174	872	1210	1010	2000	70	63	85	79	86
	Minimum	443	367	137	362	822	32	30	15	33	33
	Average	739	602	515	656	1153	49	43	40	55	65
Phosphorus	Maximum	887	1069	875	1355	1150	73	83	89	130	74
	Minimum	585	850	527	663	814	46	57	45	72	47
	Average	708	629	669	1042	991	56	70	63	101	66
Potassium	Maximum	1400	1770	1600	1300	1800	91	117	115	111	74
	Minimum	756	800	482	607	461	45	52	38	52	32
	Average	1096	1348	1097	1030	1084	70	90	82	80	53
Calcium	Maximum	68.0	49.2	69.0			7 1	6 0	7 8		
	Minimum	17 6	17.8	14.2			2 3	2 1	2 3		
	Average	36 9	30 9	34.6			4 5	4 0	4 7		
Magnesium	Maximum	135 0	129.7	116 3			25 7	27.5	27.2		
	Minimum	56 2	64.2	55.9			10 6	11 9	13 1		
	Average	93 7	103.2	83.8			18.9	22.0	19 6		
Total base	Maximum	928	901	886	657	1197	219	239	215	220	240
	Minimum	451	437	333	407	365	118	131	84	122	97
	Average	630	628	579	567	838	156	163	165	172	169

the relative amount may be little changed. It is evident, however, that even slight changes in water content are accompanied by appreciable changes in the percentage of solids.

In eleven out of sixteen comparisons made, the right ventricle contained a higher percentage of water than the left ventricle.

The gastrocnemius muscles showed the greatest variation in water content, which is not surprising in view of the fact that some of the muscles were edematous.   The livers had about the same average water content as skeletal muscle.   In thirteen out of fourteen cases the kidney tissue showed the highest water content of the five tissues studied.   The average percentage of water in the kidneys is thus definitely and significantly higher than that in any of the other four tissues.

Chloride—The chloride content of the right ventricle was higher than that of the left in eleven out of thirteen cases.   In general, the right ventricle was also richer than the left in calcium, while the opposite was true for total phosphorus, total base, potassium, and magnesium.

Skeletal muscle showed wide variations in content of chloride, the lowest being 39, the highest 225, and the average 111 mg. per 100 gm. of fresh tissue.   In the individual cases, skeletal muscle showed the lowest chloride content of the five tissues in eleven out of fifteen cases.   This is in agreement with the findings of Müller and Quincke (8), Vlădesco (17), Blum and Broun (1), and Cameron and Walton (3).

In nine out of thirteen cases the kidney showed the highest chloride content.   This also agrees with the findings of the above authors.

One person with secondary anemia (Case 8) had a considerably higher chloride content in all five tissues than the average for the corresponding group.

Phosphorus—In every instance there was more phosphorus in the left ventricle than in the right, the averages being 172 and 141 mg. per 100 gm. of fresh tissue.   Skeletal muscle gave no such consistent results; in five cases the phosphorus was lower than in either of the ventricles, in five cases it fell between the ventricles in concentration, and in four instances it was higher than in either ventricle.   The average phosphorus content of the gastrocnemius muscles was 150 mg. per 100 gm. of fresh tissue.   Phosphorus, like potassium, is apparently an intracellular element, little being present in the extracellular fluids.

Our phosphorus values for the five tissues studied agree in general with the results of Francis and Trowbridge (5) for the corresponding beef tissues, and our phosphorus values for the left ventricle are in agreement with those of Scott (11–14).

Potassium—The literature contains several reports on the potassium content of certain human tissues. Our values for potassium agree in general with the averages of others with the exception of Scott (11–13), whose average values for the left ventricle are much lower than ours; however, our values agree much more closely with those given in a later report by Scott (14).

Without exception (both in fresh tissue and in milli-equivalents per kilo of tissue water) the potassium content of the left ventricle was higher than that of the corresponding right ventricle. The average potassium content of the left ventricles was about 27 per cent higher than for the right ventricles.

As with the other elements, skeletal muscle showed marked variations in potassium. The average was 246 mg. per 100 gm. of fresh tissue and 82 milli-equivalents per kilo of muscle water.

TABLE V

Phosphorus-Potassium Ratios

| | mM P per kilo tissue water / mM K per kilo tissue water | | | | |
	Right ventricle	Left ventricle	Gastroc-nemius	Liver	Kidney
Maximum	1 13	1 17	2 10	1 62	2.20
Minimum	0 67	0 56	0 54	0 86	0 85
Average ...	0 82	0.80	0 85	1 30	1 34

The potassium content of the livers and kidneys varied widely, the average for the kidneys being the lowest of the five tissues studied. In this connection, it may be noted that the kidneys had the highest averages for water, chlorides, and total base. Norn's findings (9) indicate that kidney tissue is rich in sodium. The findings for the potassium content of all five tissues agree in general with those of Calhoun *et al.*(2).

Phosphorus-Potassium Ratios—As shown in Table V, there is a marked constancy in the P:K ratios for a given kind of tissue. The highest P:K ratios occurred in the liver and kidney, while those for skeletal muscle and right and left ventricle were in close agreement.

Calcium—Calcium was usually more concentrated in the right

ventricle than in the left, the averages being 7.3 and 6.4 mg. per 100 gm. of fresh tissue for the right and left ventricles respectively.

In the skeletal muscles the calcium figures showed wide variations, the two extremes being 12.7 and 3.6 mg. per 100 gm. of fresh tissue. The average was 7.4, which is in close agreement with the results of Katz (7).

Calcium determinations were not made on liver and kidney.

Magnesium—With four exceptions, the magnesium content of the left ventricles was higher than that of the right. The average for the right was 18.4 and for the left 21.1 mg. per 100 gm. of fresh tissue.

For the gastrocnemius muscles the average was 18.4 mg. per 100 gm. of fresh tissue, which is in close agreement with the results of Katz. Magnesium determinations were not made on liver and kidney.

Total Base—When expressed in milli-equivalents per kilo of tissue water, the left ventricle contains more base than the right in all but three cases, the average being about 4.5 per cent higher for the former than for the latter. The left ventricles contained an average of about 5 per cent more solid material than did the right ventricles, the average amount of water being about 1.3 per cent greater in the right than in the left.

Gamble, Ross, and Tisdall (6) have expressed the belief that probably most of the soft tissues of the human body have about the same osmolar concentration of total base in the tissue fluids. The figures reported here support this belief.

Van Slyke, Wu, and McLean (16), in a typical case, found 162 and 174 milli-equivalents of base per kilo of serum water and cell water respectively. Values close to these have been calculated by Peters and Van Slyke for edema fluid and muscle water.

The average total base figures (in milli-equivalents per kilo of tissue water) for the five tissues used in the present study all fall within the range of 164 ± 8, the lowest being 156 for the right ventricles, and the highest being 172 for the livers.

It is interesting that in tissues, as in red blood cells and serum, the individual bases may show such marked differences while the total base content falls within the same general range.

SUMMARY

Samples of right ventricle, left ventricle, gastrocnemius, liver, and kidney obtained from nineteen autopsy cases were analyzed for water, chlorides, phosphorus, potassium, and total base. Right ventricles, left ventricles, and skeletal muscle were in addition analyzed for calcium and magnesium.

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STUDIES ON HEPARIN

I. THE PREPARATION OF HEPARIN

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The observation that an extract of dog liver contains a substance which retards the coagulation of blood *in vitro* was first made by McLean in 1916 (1). In 1918 Howell and Holt (2) named this substance "heparin" and described a method for its preparation from dried dog liver. In 1923 Howell (3) published a much improved method of preparation and defined the unit of heparin as being that weight of substance which would prevent 1 cc. of cat blood from clotting for 24 hours when kept in the cold.

Because of the importance of heparin in certain physiological experiments, and since the cost of preparing this material from dog liver was high, an attempt to secure the anticoagulant from a cheaper and more readily available source was initiated by Professor C. H. Best, in these Laboratories. The first experiments were carried out by Dr. E. W. McHenry and Dr. D. Glaister. These investigators were able to prepare small quantities of an anticoagulant from beef liver. In their experiments the Howell method was used. Later one of the present authors (A. F. C.) working in the Department of Physiology, confirmed their results. The problem was again taken up in these Laboratories by the authors and a process has been developed by which several hundred gm. of heparin have been prepared from beef liver.

The essential features of the Howell method of preparing heparin from dog liver are: (1) mincing and drying the liver, (2) alkaline aqueous extraction, (3) precipitation with acetone, (4) extraction with hot methyl alcohol, (5) precipitation with acetone. This method is quite satisfactory for dog liver, particularly when only small quantities of tissue are used. When larger amounts of material are used, however, this process is tedious, particularly

when beef liver is used. Perhaps the most undesirable feature of the process is the filtration of the alkaline aqueous extract. Our experience has been that it is almost impossible to clarify the extract obtained at this stage. In the process which we have devised, sufficient ammonium sulfate is used in the extraction medium to precipitate most of the protein-like materials. This gives a product which is readily filtrable. It is also desirable to eliminate the preliminary drying of the liver recommended by Howell. In our process we have used either fresh liver or liver which has undergone autolysis for 24 hours after mincing.

<div align="center">EXPERIMENTAL</div>

We need not discuss here in detail the experimental work which was entailed in the development of our method of preparing heparin from beef liver. The method which we have adopted works quite satisfactorily with 100 pound quantities of liver, and is as follows:

100 pounds of fresh beef liver are minced. In certain experiments this tissue was immediately extracted with 62 liters of 0.5 N sodium hydroxide and 7.5 liters of saturated ammonium sulfate. In other experiments the tissue was allowed to autolyze for 24 hours before extraction. The mixture is slowly heated with constant stirring to 50° in a steam-jacketed vessel, and maintained at this temperature for 1 hour. At this temperature no coagulation occurs and the heparin is extracted from the tissue. The protein is coagulated by heating rapidly to 70°. The mixture is filtered while hot, yielding about 40 liters of dark brown filtrate. This is then acidified to pH 2 to 2.5 with concentrated sulfuric acid. This precipitates most of the heparin. The acid mixture is then heated to 60° and filtered through linen cloths and the precipitate washed with 20 liters of hot dilute sulfuric acid (pH 2 to 2.5). This removes traces of ammonium sulfate which may be undesirable in the later stages of purification. The precipitate so obtained is suspended in 6 liters of 95 per cent ethyl alcohol for 20 hours. This treatment removes a brown oily substance. The alcohol is then decanted; the precipitate centrifuged and dissolved in 6 liters of water. The reaction is adjusted to pH 8 and 5 cc. are removed for potency estimations. To the rest of the solution, 25 gm. of trypsin in aqueous suspension and 10 cc. of xylene are

added.   The flask is placed in an incubator at 36° for 36 hours.
Every 12 hours the solution is readjusted to pH 8.   After digestion
another 5 cc. sample is removed, heated to 75° to destroy the
trypsin, and the potency determined.   To the remainder of the
digest 12 liters of 95 per cent ethyl alcohol are added and the
mixture is made slightly acid (litmus) with HCl.   After standing
for 24 hours the precipitate settles quite firmly to the bottom of
the flask, leaving the clear supernatant liquid free from heparin.
The precipitate is removed and added to 1.5 liters of water con-
taining just enough 0.5 N sodium hydroxide to dissolve it.   This
solution is heated to 75° to destroy any trypsin present, and after
cooling is centrifuged to remove a small amount of insoluble
material.   The anticoagulant is again precipitated by the addition
of 2 volumes of acetone and the mixture made slightly acid (lit-
mus) with HCl.   After standing for 15 hours, the precipitate is
centrifuged, washed with 95 per cent ethyl alcohol, dried at room
temperature, and assayed.   The heparin so prepared contains
approximately 3.5 units per mg.

The results of several experiments with 100 pound quantities of
minced beef liver are shown in Table I.   In the results of the
experiments listed in the first part of Table I the liver was imme-
diately extracted, and in the second part the liver was allowed to
autolyze for 24 hours before it was extracted.

In order to increase the potency of the substance and secure a
product of uniform composition, the following process is used.

The crude material obtained from several 100 pound lots of liver
is combined and weighed.   To every 100 gm. of heparin, 5.0
liters of water are added.   This does not produce a clear solution
as some insoluble material is still present.   Ammonium sulfate is
added to give a concentration of 0.8 per cent.   The acidity is then
adjusted to pH 4.1 to 4.3 with N H_2SO_4.   After heating at 70° for
1 hour, a dark brown precipitate settles.   This is removed by
filtration and 1.5 volumes of acetone are added to the filtrate to
precipitate the heparin.   After standing for 15 hours, the acetone
is decanted.   The precipitate is washed several times with ethyl
alcohol and dried *in vacuo*.   The product obtained in this manner
dissolves readily in water and contains approximately 5 units per
mg.   If heparin of a slightly higher unit content is required this
product may be further purified with Lloyd's reagent as elaborated

by Howell (4) or fractionated with acetone as described by us in a later paper (5).

The heparin preparations were assayed physiologically by determining their anticoagulant effect on cat blood. A known amount of the preparation to be tested was dissolved in isotonic saline. A similar solution was prepared from a standard heparin

TABLE I

Yields of Heparin Obtained at Different Stages of Purification

100 pounds of liver were used for each experiment.

Experiment No.	Treatment	Heparin			Heparin
		Before trypsin	After trypsin	After acetone	
		units	*units*	*units*	*gm.*
1	Extracted immediately			52,500	15
2				48,700	6.5
3				74,000	20
4		50,000	50,000	70,000	20
5		45,000	45,000	60,000	19
6		46,000	46,000	86,000	24
Average.........		47,000	47,000	65,000	17.5
1	Autolyzed 24 hrs.			180,000	50
2		100,000	100,000	124,000	31
3		70,000	70,000	259,000	74
4		70,000	70,000	220,000	70
5		80,000	80,000	240,000	80
6				168,000	42
7		50,000	50,000	175,000	50
8		60,000	60,000	170,000	50
Average..........		72,000	72,000	192,000	56

powder.[1] By comparing the effects of the two solutions on the arterial blood of cats, the potency of the unknown material was estimated.

[1] The first standard used was a Hynson, Westcott and Dunning powder containing 5 units per mg. We are establishing, for our own convenience, a standard material containing approximately 15 units per mg.

DISCUSSION

A method is described for preparing quantities of heparin from beef liver. In this process some of the time-consuming features of Howell's process for preparing heparin from dog liver are eliminated. A summary of the yields of crude heparin obtained from 100 pound quantities of beef liver is given in Table I. From this table some interesting observations may be made. In the first place, the figures show that beef liver contains appreciable quantities of this anticoagulant. Secondly, heparin is not destroyed by trypsin. Thirdly, a much greater quantity of heparin is obtained when the liver is allowed to autolyze for 24 hours before extraction. In view of this increased yield of autolysis, it seems possible that heparin may exist in liver in a combined form, and that it is set free during autolysis. Finally, it will be observed that the total number of units found in the tryptic digest is much less than that obtained in the purified product. This is particularly well illustrated in the figures listed in the second part of Table I. Here the average unit content per pound of liver after tryptic hydrolysis is 720, whereas the unit content of purified product per pound of liver is 1920. This suggests that in the crude preparations there may be one or more substances which act as blood coagulants. Cephalin or other lipids are probably responsible for this effect since the coagulant is largely removed by acetone and alcohol.

SUMMARY

1. A method for preparing heparin from beef liver is described.

2. Larger yields of heparin are obtained from autolyzed liver than from fresh tissue.

3. Heparin is not destroyed by trypsin.

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STUDIES ON HEPARIN

II. HEPARIN IN VARIOUS TISSUES

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In a former communication (1) a method for preparing large quantities of heparin from beef liver was outlined. By the use of this process fairly consistent results were obtained both with regard to the purity and to the quantity of heparin extracted from different lots of liver. It was thought that, from this method of preparation, a modified form could be developed which would be suitable for use with much smaller quantities of material. Hence, some interesting information might then be obtained by a study of the heparin content of various tissues.

The occurrence of heparin in extracts of dog liver was first demonstrated by Howell and Holt (2). Further, they reported its presence in extracts of the lymph glands and in blood. King (3) obtained it from the uterine mucous membrane and Kashiwamura and Katsuki (4) state that they found this anticoagulant in extracts of milk, kidney, lung, and other tissues.

While a review of the literature suggests that heparin is fairly widely distributed in the animal body, yet no data are given by the various investigators concerning the amounts found by them, or the purity of their preparations. Accordingly, in the present investigation, by using a standard method of preparation, we have attempted to ascertain the relative amounts of this substance and the purity of the products obtained from various tissues.

EXPERIMENTAL

We have endeavored to determine the heparin content of the following tissues: ox liver, dog liver, hog liver, ox spleen, ox heart, ox thymus, ox lung, ox muscle, ox blood, and ox serum. In the experiment with blood, sodium citrate was added to prevent

coagulation. The blood serum was obtained by allowing blood to stand in the refrigerator for 4 days and then decanting the serum. A series of experiments was also conducted with beef liver to which was added a quantity of ethyl acetate and toluene. It is well known that, if these substances are added to yeast before extraction, a much higher yield of certain enzymes is obtained.

The method which we have used for preparing heparin from the various tissues is very similar to our former process for obtaining quantities of this material from beef liver. From a series of experiments with beef liver, it was found that the present method gave results with 500 gm. of this tissue which were remarkably constant both in respect to the yield of heparin and to the purity of the product. The heparin content of each tissue was determined in triplicate experiments. The method was as follows:

500 gm. of fresh tissue were finely minced and autolyzed at 25° for 24 hours. To this were then added 730 cc. of 0.5 N NaOH and 87 cc. of saturated ammonium sulfate solution. The mixture was extracted at 50° for 30 minutes and then rapidly heated to 70°. It was filtered immediately through linen cloths and allowed to drain for 12 hours. The filtrate was acidified to pH 2 to 2.5 with concentrated sulfuric acid and heated to 65°. The hot mixture was filtered and the precipitate washed with hot dilute sulfuric acid (pH 2 to 2.5) to remove traces of ammonium sulfate. The precipitate was extracted at room temperature with 100 cc. of 95 per cent ethyl alcohol for 20 hours. The alcohol was removed by centrifugation and the precipitate dissolved in 75 cc. of alkaline water (pH 8 to 8.4). 2 gm. of trypsin and a few drops of xylene were added. The solution was incubated at 37° for 36 hours. During this time adjustments in pH were made when necessary. At the end of the digestion period, 2 volumes of 95 per cent alcohol were added. The mixture was acidified with 0.5 cc. of HCl and allowed to stand for 20 hours. The precipitated heparin was separated by centrifugation and dissolved in 50 cc. of water at pH 8. The alkaline solution was heated to 75° to prevent subsequent tryptic action. An insoluble fraction was removed by centrifugation. To the clear supernatant solution, 2 volumes of acetone were added and the mixture was acidified with 0.3 cc. of HCl. The precipitate which formed was separated by centrifugation, washed with alcohol, and dried *in vacuo*. This dried powder was

weighed and was designated "crude heparin."   A sample of this was assayed, and the number of units per kilo of tissue calculated. The remaining powder was dissolved in 0.8 per cent ammonium sulfate (50 cc. per gm. of powder).   The acidity was adjusted to pH 4.1 to 4.3 with N sulfuric acid and the solution heated to 70°. A precipitate formed and was removed by filtration.   The heparin in the filtrate was precipitated by adding 1.5 volumes of acetone. After standing for 15 hours the acetone was decanted and the precipitate dissolved in water.   The volume used here was twice

TABLE I

Distribution of Heparin in Various Tissues

Tissue	Crude heparin per kilo tissue		Purified heparin per kilo tissue	
	mg.	*units*	*mg.*	*units*
Ox liver.............	780	1800	190	1900
" " •............... ...	880	2000	315	1900
" spleen...............	1000	1030	230	700
" heart...	200	420	54	380
" blood....................	260	185	66	60
" thymus.....	640	70	310	35
" blood serum...	24	4		
" lung	840	3300	230	2200
" muscle..	2000	1500	600	1900
Hog liver......	1400	5000	340	1700
Dog "	900	4500	330	4400

* In these experiments 7.5 cc. of ethyl acetate and 35 cc. of toluene were intimately mixed with each 500 gm. of minced tissue.   The mixture was then autolyzed for 24 hours and the heparin purified by the standard procedure.

that used in dissolving the crude heparin.   Glacial acetic acid was then added to give a concentration of 2 per cent acetic acid, and Lloyd's reagent mixed with the solution (500 mg. per 50 cc. of solution).   The mixture was allowed to stand for 12 hours.   The precipitate was removed and 2 volumes of acetone added to the clear supernatant liquid.   The white precipitate was separated by centrifugation, washed with alcohol, and dried *in vacuo*.   This was designated "purified heparin" and was weighed, assayed, and the units per kilo of tissue calculated.

In determining the number of units of heparin contained in the crude and the purified preparation of the various tissues, we have adopted the Howell unit as being that weight of heparin which would prevent the clotting of 1 cc. of cat blood when kept in the cold for 24 hours. Since the blood of different cats varies considerably in the rate of clotting, the heparin preparation of unknown strength was always compared with a standard heparin. By this method of assay the variations in the different tests were largely overcome. In Table I are listed the number of units of heparin obtained from each tissue. The value given for each tissue is the average figure obtained from three different experiments. It was found that for any particular tissue the amounts of heparin obtained in the triplicate experiments agreed remarkably well, the maximum deviation being not over 20 per cent.

DISCUSSION

In the present investigation we have attempted to ascertain the heparin content of various tissues. In this work the same method of preparation was used in all experiments. The amount of heparin and the number of units obtained per kilo of tissue are listed in Table I. The values given are the average results obtained from three 500 gm. samples of each tissue.

Some interesting observations may be made on the basis of the values listed in Table I. In the first place, a blood anticoagulant was found in all the tissues examined. In certain tissues, *i.e.*, muscle and lung, the amounts obtained were of the same order as the quantities found in liver. This observation would indicate that the liver is not necessarily the seat of production for heparin, nor does it contain the only reserve of heparin for the body. The high yield obtained from the liver and the muscle might suggest glycogen as a factor in the production of heparin in the body. However, the relatively large amounts of this substance found in the lung somewhat vitiate this theory. The quantity of heparin found in the spleen was about one-third that found in beef liver. The heart, thymus, and blood contained much smaller amounts of heparin and the blood serum contained an almost negligible quantity. Secondly, in most experiments a little loss of heparin was encountered during the later stages of purification. Thirdly, the addition of ethyl acetate and toluene to beef liver during

autolysis did not increase the yield of heparin. Finally, the experiments with dog liver indicated that it contained approximately twice as much heparin as beef liver.

SUMMARY

The presence of a blood anticoagulant, presumably heparin, has been demonstrated in various tissues. It was found in greatest quantities in muscle, liver, and lung. Much smaller quantities were obtained from heart, thymus, spleen, and blood. Dog liver contains approximately twice as much heparin as does beef liver.

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STUDIES ON HEPARIN

III. THE PURIFICATION OF HEPARIN

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In view of the fact that we had succeeded in preparing a quantity of heparin from beef liver (1), it was decided to continue work on the purification of this substance with the object of isolating it in pure form. While we have had some success in this direction in that we have obtained a product which contains approximately 500 units per mg. and which is uniformly microcrystalline, yet, because of the high ash content of the crystalline product and also because of the appearance of the crystals themselves, we are not as yet prepared to state that our final product is heparin itself.

Previous investigators have used various methods to increase the activity of heparin preparations. Howell (2) precipitated much of the inactive material in crude heparin by means of cadmium chloride and obtained a very active product, 1 mg. of which prevented the clotting of 40 cc. of blood. This product gave negative tests for protein, sulfur, and phosphorus. However, by this method of purification great losses occurred, and only about 20 per cent of the heparin was recovered in the highly active form. In a later paper (3) a much greater purification of heparin was effected by the use of Lloyd's reagent and by barium hydroxide. 1 mg. of this product prevented the clotting of 100 cc. of blood. This product was nitrogen- and phosphorus-free. He concluded from various chemical reactions that heparin was probably a derivative of glucuronic acid. We have reported (4) a product containing 200 units per mg. This material was purified mainly by organic solvents, *i.e.* acetone and pyridine. More recently Schmitz and Fischer (5) have isolated the brucine salt of heparin. They stated that their product was 32 times as active as their original material. From a combustion analysis they determined the formula for heparin to be $C_{18}H_{22}O_{17}6H_2O$.

In attempting to purify crude heparin we first tried the method suggested by Howell. We found that a purification could be effected by Lloyd's reagent, and that the potency could be increased from 5 units to approximately 15 units per mg. The method of purification with cadmium chloride yielded a much more active product, but, as in Howell's experiments, there was a great loss of potency. The experiments with barium hydroxide always resulted in a great loss of heparin and little purification was effected.

Since the attempts to purify heparin with inorganic substances always resulted in a very poor yield of active material and in a product with a very high ash content, it was decided to attempt a purification mainly by the use of organic solvents. By fractionation experiments in which acetone and pyridine were mainly used, a product was obtained which contained 200 units of heparin per mg. This product contained needle-like crystals, which we were never able to separate from the gummy amorphous material. However, on this very active product some interesting observations were made which have been of the greatest aid in the more recent studies. In the first place, the product was surprisingly stable to oxidizing or reducing agents in a neutral or an alkaline solution. Secondly, in alcohol the ammonium salt was soluble, whereas the sodium salt was insoluble. This difference in solubility would suggest that definite derivatives had been formed. From this it seemed reasonable to suppose that heparin contains a free carboxyl group. Thirdly, the product contained 3.2 per cent nitrogen, and after mild treatment with nitrous acid or sodium nitrite in acetic acid solution the physiological activity completely disappeared. This observation suggested that heparin probably contained a free NH_2 group. Thus heparin would appear to be an amphoteric substance. Finally, fractionation experiments indicated that the point of minimum dissociation was about pH 5. In the later work on the purification of heparin we have made constant use of this fact, and it has contributed greatly to the success of the experiments. It was found that much foreign material could be separated at this acidity by means of organic solvents or inorganic substances, with little loss of activity.

It seemed desirable, before commencing work on the purification of heparin, to ascertain how stable this substance was to acid and to alkali. This was done by dissolving heparin (14 units per mg.) in acid or alkali of known strength, and heating the solutions at 80° for various periods of time. The solutions were then neutralized and physiologically assayed. The results of two series of these experiments are shown graphically in Figs. 1 and 2. It
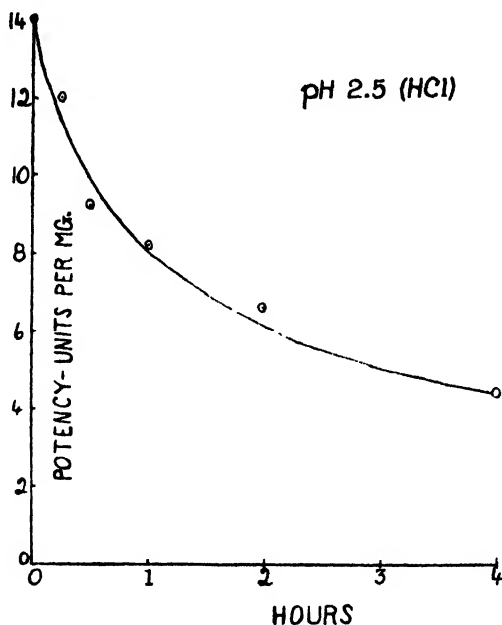
FIG. 1. Rate of acid hydrolysis at 80°

will be seen from these graphs that heparin is destroyed by acid and by alkali. This destruction proceeds much more rapidly in acid than in alkali.

In numerous experiments with various solvents and inorganic substances, we have obtained very active preparations of heparin. However, in the present paper we shall describe in detail only that method of purification which yields the most active product with the minimum loss of potency.

Purification—The first step in the purification of heparin (5

units per mg.) was the fractionation with Lloyd's reagent as described by Howell (3). 20 gm. of heparin were dissolved in 3 liters of water. To this were added 60 cc. of glacial acetic acid and 30 gm. of Lloyd's reagent. The mixture was allowed to stand for 12 hours. The precipitate was then removed by filtration on a Buchner funnel. The filtrate was clear and pale yellow in appearance. To this were added 6 liters of acetone and the mixture allowed to stand overnight. The precipitated heparin was then

0.25 N ALKALI (NaOH)
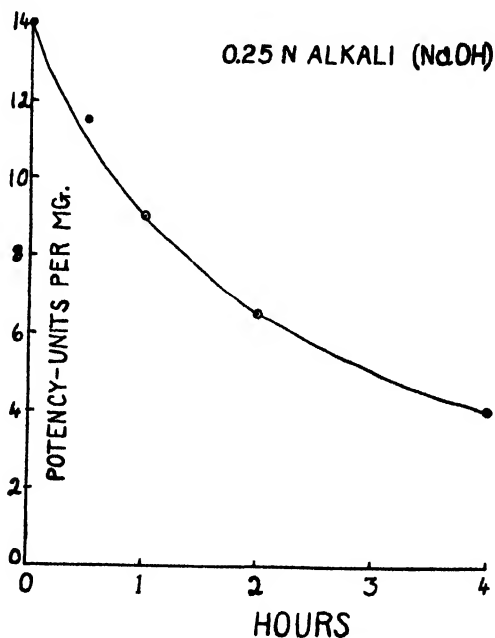
Fig. 2. Rate of alkali hydrolysis at 80°

centrifuged off and washed twice with alcohol. It was then dried *in vacuo*. The resultant white powder contained 15 units per mg. By this process 97 per cent of the original potency was recovered. Further purification was carried out on 1 gm. quantities of this material in the following manner. 1 gm. of heparin was dissolved in 100 cc. of water and the acidity adjusted to pH 5.0 with N HCl. To this solution were added 4 cc. of a 10 per cent solution of cadmium chloride. The mixture was shaken and allowed to stand at room temperature for 24 hours, after which it

was centrifuged and washed with 10 cc. of a 0.4 per cent solution of cadmium chloride. The washings after centrifugation were combined with the main volume of the liquid. After drying, the precipitate weighed 750 mg. and contained less than 2 per cent of the original activity. The supernatant liquid was transferred to an Erlenmeyer flask and 50 cc. of acetone were added slowly with constant stirring. A precipitate readily formed and was allowed to stand overnight at room temperature. The mixture was then centrifuged and the precipitate dried. It weighed 175 mg. and contained less than 5 per cent of the original activity. The clear supernatant liquid was then divided between two 250 cc. centrifuge tubes, and 200 mg. of sodium chloride were added to each tube. After the salt had dissolved, 80 cc. of acetone and 0.3 cc. of N HCl were added to each tube. The tubes were then placed in a refrigerator overnight. The following morning they were centrifuged and both precipitates dissolved in 15 cc. of isotonic saline. The heparin was again precipitated by the addition of 30 cc. of acetone. After standing in the refrigerator overnight, the mixture was centrifuged. The dried precipitate weighed 70 mg. It was dissolved in 10 cc. of water. The solution contained a small amount of coloring material which was removed by oxidation with superoxol. This was done by making the solution slightly alkaline by the addition of 3 drops of N NaOH, and adding to this 0.6 cc. of a 1:1 dilution of superoxol. It was allowed to stand at room temperature for 1 hour and was then heated in a water bath at 50° for half an hour. After cooling to room temperature, the acidity was adjusted to pH 5 and 80 mg. of sodium chloride were added. The heparin was then precipitated with 20 cc. of acetone. After standing in the refrigerator overnight, the mixture was centrifuged, and the precipitate again dissolved in 10 cc. of water. At this stage tests were made for inorganic substances. The test with hydrogen sulfide on an ammoniacal solution was negative. The test for calcium, however, was distinctly positive. The calcium was removed by chilling the solution in an ice bath and adding 0.5 cc. of a 2 per cent solution of oxalic acid. The mixture was allowed to stand in the ice bath for 6 hours. The calcium oxalate was then removed by centrifugation. The supernatant solution was again made isotonic with sodium chloride and the heparin precipitated by adding 2 volumes of acetone. After

standing overnight the heparin was centrifuged off and dried.   At
this point the purified heparin from three such lots was combined
and dissolved in 10 cc. of isotonic saline.   The acidity was adjusted
to pH 5 and 5 cc. of acetone were added.   The solution developed
a slight turbidity and was allowed to stand at room temperature
for 24 hours.   The small precipitate which settled was then
centrifuged off.   To the supernatant solution were added 10 cc. of
acetone and the mixture was placed in the refrigerator overnight.
The precipitated heparin was then centrifuged off, dried, and
divided into three fractions.   One fraction was precipitated from
alcohol as the sodium salt.   Another fraction was precipitated as
the hydrochloride, and the third fraction retained as the free
substance (Sample A).

Fig. 3. A microcrystalline preparation of heparin obtained from acid
alcohol.

The sodium salt was prepared as follows: 40 mg. of the purified
heparin were dissolved in 4 cc. of carbonate-free 0.1 N NaOH.   To
this were added, slowly, 8 cc. of absolute ethyl alcohol.   The
mixture was placed in the refrigerator for 4 hours.   The precipi-
tated sodium salt was then centrifuged off, and washed with 4 cc.
of absolute ethyl alcohol.   It was then dried *in vacuo* (Sample B).
The hydrochloride was prepared as follows: 40 mg. of the puri-
fied heparin were dissolved in 4 cc. of 0.4 per cent sodium chloride.
To this solution was added 0.4 cc. of N HCl.   Ethyl alcohol made
0.1 N with hydrochloric acid was then added until the first sign of
turbidity appeared.   This required 7.5 cc. of acid alcohol.   The
solution was then allowed to cool slowly in the refrigerator, during

which time it became more turbid and a microcrystalline precipitate gradually settled to the bottom of the tube. After standing in the refrigerator for 6 hours, the heparin was centrifuged off and the crystals washed with 15 cc. of ether. The ether was then decanted off and the residue dried *in vacuo* (Sample C). A photomicrograph of the crystals is shown in Fig. 3.

Samples A, B, and C were then subjected to a chemical analysis and to a biological assay. In the tabulation below are given the average values of two analyses for nitrogen, carbon, hydrogen, and ash for each sample (Samples A, B, and C).

Sample	Carbon	Hydrogen	Nitrogen	Ash
A	22.01	3.70	2.70	33.27
B	20.08	3.57	1.90	38.78
C	23.80	3.96	2.74	25.30

The ash was found to consist mainly of sodium chloride.

Miscellaneous Experiments—In view of the fact that heparin would appear to be an amphoteric substance, attempts were made to form acid and basic salts. Since heparin hydrochloride was microcrystalline in nature it was thought that perhaps better crystals might be obtained by using other acids. Accordingly, with another sample of purified heparin, an attempt was made to form heparin picrate. This experiment was conducted in a manner similar to the hydrochloride experiment, except that picric acid and picric acid alcohol were used instead of hydrochloric acid. In this experiment a crystalline precipitate formed which, microscopically, appeared very similar to the hydrochloride crystals. We also tried to form heparin sulfate and heparin phosphate with little success.

Experiments designed to form a crystalline basic salt of heparin were also conducted. We attempted to prepare the lead, barium, and sodium salts. These salts were always amorphous in character. In an endeavor to form the ammonium salt, concentrated ammonia was added to an alcoholic solution of heparin. No precipitate formed. Since heparin would appear to be weakly acidic in character we also tried to form the ammonium salt according to the method of Bateman and Conrad (6) by bubbling

dry ammonia gas into heparin dissolved in 99 per cent ethyl alcohol. No precipitate appeared. We are now inclined to think that the failure of this experiment may have been due to the presence of inorganic materials in the heparin.

We have also tried to purify the heparin further by fractionation experiments with various solvents. For this purpose we used glacial acetic acid, ethyl acetate, methyl alcohol, butyl alcohol, amyl alcohol, pyridine, and chloroform. With these solvents no further purification was effected.

Certain color tests were also carried out on the purified heparin. The color reaction for carbohydrate with α-naphthol was positive. The iodine test for glycogen was negative. The tests with orcinol, phloroglucinol, and naphthoresorcinol were negative. The failure to obtain these tests was not due to the heparin solutions being too dilute to give the reactions, since they were carried out on various concentrations of heparin, the highest being 4 per cent.

Biological Assay—In an attempt to ascertain the activity of the three preparations of heparin (Samples A, B, and C), we have used the clotting of cat blood as the index of potency, and have adopted Howell's definition of the unit of heparin as being that weight of substance which will prevent 1 cc. of cat blood from clotting when kept in the cold for 24 hours. In arriving at any definite figure for the activity of a preparation, many determinations are obviously necessary as the blood of different cats varies considerably in the rate of clotting. There are other factors also to be considered, such as the kind and the amount of anesthetic used, the temperature at which the blood is kept during the test, the length of time the cat is under the anesthetic before the test is made, and also the amount of blood withdrawn during the test. Invariably the blood withdrawn near the end of a potency test shows a greater tendency to clot than do the first samples of blood removed.

We assayed the three heparin preparations in the following manner. A sample of each preparation was weighed and dissolved in 40.0 cc. of isotonic saline. The weights of the heparin samples were: Sample A 3.90 mg., Sample B 4.24 mg., Sample C 4.28 mg. Three 1.0 cc. samples of each of these solutions were removed and diluted with isotonic saline to 10, 20, and 30 cc. Each sample of heparin was tested at these three dilutions. 0.3 cc. of these solutions was pipetted into small flocculation tubes which

were graduated at 1 cc.   0.7 cc. of cat blood was then run into each of the nine tubes containing the known amount of heparin. The cats used in these tests weighed 2 kilos and were anesthetized by injecting a solution of sodium amytal into the peritoneal cavity.   The amount of amytal used was 80 mg. per kilo of body weight.   Blood was secured from a cannula inserted into the carotid artery.   After the blood had been added to the tubes they were stoppered and inverted twice to insure the mixing of the heparin with the blood.   They were then placed in an ice bath

TABLE I

Results of Biological Assays of Three Purified Heparin Preparations

Cat No.	Sample A	Sample B	Sample C
	units per mg.	*units per mg.*	*units per mg.*
1	430	395	395
2	360	395	395
3	430	395	395
4	430	330	330
5	360	330	330
6	430	550	550
7	430	550	440
8	360	550	550
9	360	550	550
10	480	550	550
11	600	395	395
12	430	615	615
13	430	550	550
14	360	615	550
Average.........	421	484	471

for 24 hours.   At the end of this time they were examined and the results recorded.

In the interpretation of the results there was often some difficulty in assigning a definite figure for the activity of a preparation. Without exception, the blood in the tubes containing the most heparin (dilution 1:10) flowed quite freely at the end of 24 hours. The blood containing a dilution of heparin 1:20 in some tubes flowed freely at the end of the test, in other tubes it had clotted, and in most tubes it flowed but not freely.   When a tube had

clotted at this dilution and flowed freely at a 1:10 dilution we assigned a potency value to the particular sample of heparin midway between these dilutions. When the test at this dilution (1:20) was slightly doubtful, a value of 80 per cent of the difference between the first and the second dilution was added to the first dilution, and this value given for the activity of the particular sample of heparin on test. The samples of blood with the highest dilution of heparin (1:30) in most tests had either clotted or would not flow freely. When the dilution (1:20) showed no signs of clotting and a dilution (1:30) had clotted, a value for the activity of the preparation was given as midway between these dilutions. If the highest dilution was doubtful a value of 80 per cent of the difference between the second and third dilution was added to the second dilution and this value taken as the activity of the preparation on test. In Table I are listed the values found for the activity of the three samples of heparin (Samples A, B, and C) on fourteen cats.

<div style="text-align:center">DISCUSSION</div>

In the present communication we have outlined methods for the preparation of heparin in three forms. One sample was prepared as the neutral substance, another as the sodium salt, and another as the hydrochloride. Each of these samples was subjected to a physiological assay and to a chemical analysis. The results of the biological assay indicate that the preparations contained between 400 and 500 units per mg. The average potency value for the three samples is 459. If a correction is made for the ash, which was shown to be mainly sodium chloride, the average value is 684 units per mg. The chemical analysis showed that in addition to carbon and hydrogen the products contained small amounts of nitrogen. The ash content is very appreciable in all the samples, and is particularly high in the sample prepared as the sodium salt. This is rather surprising since it was precipitated from an aqueous alkaline solution with alcohol. Further, this precipitate was washed with alcohol to remove traces of sodium hydroxide.

The sample of heparin that was prepared as the hydrochloride was microcrystalline in nature. The crystals generally appeared in small rosettes. They were obtained by adding acid alcohol to an acid solution of heparin containing a small amount of sodium

chloride. It is well known that the presence of small amounts of sodium chloride aids in the crystallization of certain substances, *e.g.* certain sugars and amino acids. At first we were inclined to believe that we had isolated heparin hydrochloride in crystalline form. However, from the high ash content of this material and from the appearance of the crystals themselves, we are now inclined to think that a more active heparin is obtainable.

There are certain findings in the present work which are not in accord with the work of previous investigators. Howell, from color reactions with orcinol and naphthoresorcinol, concluded that heparin was a derivative of glucuronic acid. These tests were negative even in a 4 per cent concentration of our purified heparin. In regard to the more recent work of Schmitz and Fischer, who claim to have isolated the brucine salt of heparin, and have assigned to heparin the formula $C_{18}H_{32}O_{17}6H_2O$, we wish to point out certain facts which do not coincide with our findings. In the first place, this product gave positive tests with orcinol and naphthoresorcinol. Secondly, they report a nitrogen-free material. Our most active preparation contained nitrogen and probably amino nitrogen, since the physiological activity was readily destroyed by treating an aqueous solution of the heparin with nitrous acid. Thirdly, they do not express the activity of their crystalline product in terms of the Howell unit, but state that they obtained a product which was 32 times as active as their starting material. Our product contained approximately 500 units per mg. and is 100 times as active as our original material. Finally, it may be noted that it is of the utmost importance to secure the crystals completely free from any amorphous material. Several times in the preliminary work on the purification of this substance we were led to believe that some well formed crystals were heparin. However, upon carefully removing the amorphous material it was found that the high physiological activity was due to this material and not to the crystals themselves.

SUMMARY

1. Methods are given for obtaining very active preparations of heparin.

2. The physiological activity of three preparations of heparin gave an average value of 459 units per mg.

3. The combustion analyses on these products show that they contain nitrogen in addition to carbon and hydrogen. The ash content in all samples was high.

4. The purified heparin gave a positive reaction with α-naphthol. The tests with iodine, resorcinol, phloroglucinol, and naphthoresorcinol were negative.

We are indebted to Dr. H. Stantial for the chemical analyses, and to Dr. J. Craigie for the photomicrograph.

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THE PREPARATION OF CRYSTALLINE LACTIC ACID

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On account of its importance in intermediary metabolism, lactic acid was among the first compounds chosen in our plan, which we have described in a previous communication (1), to augment the available data on the free energies of formation of substances significant in biological chemistry. It was necessary for this purpose to obtain pure crystalline lactic acid, free of water, anhydride, and lactide. The only description in the literature of the preparation of crystalline lactic acid is that of Krafft and Dÿes (2). Table I shows that the product obtained by their method contains relatively large quantities of anhydro impurities. The subject of the present communication is the description of a method which yields the active isomers of lactic acid in a crystalline state, free of water, anhydride, and lactide, supplemented by the description of two methods of separating the active forms from the commercial syrup.[1] Lactic acid commercially available at present either is in the form of the U.S.P. syrup, which usually exhibits a low optical activity corresponding to the excess it happens to contain, which is variable, of one or the other optical isomer, or is the expensive zinc sarcolactate. The methods described below now make it possible to obtain easily and quickly and at low cost large quantities of both active isomers in a relatively high degree of purity.

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[1] In this communication the form of lactic acid commonly named sarcolactic or d-lactic acid is designated as l(+)- or l-lactic acid. The salts of this form are levo- and the free acid dextrorotatory. The opposite form is correspondingly designated as d(−)- or d-lactic acid. Optically inactive lactic acid is referred to as the dl form without any implication regarding its constitution; i.e., whether it is a simple mixture of equal quantities of the two active forms, or a definite compound.

The summary in Table III of some of the physical properties of the free acids prepared by these methods shows that they probably have not been obtained in as pure a state out of solution hitherto. For example, the melting points of the active forms are more than 25° higher than the values given in standard reference works (3, 4). They are also less hygroscopic than they are commonly described.

We shall describe first the preparation of the crystalline free acids from the commercial syrup (which contains as a rule about 50 per cent lactic acid, 30 per cent anhydride and lactide, and 15 per cent water).   Briefly, the method consists of fractional distillation followed by fractional crystallization from a mixture of equal volumes of ethyl and isopropyl ethers.   The typical procedure was as follows: 200 cc. of syrup were distilled from a 1 liter Claisen flask, first with a water pump at about 60° until most of the water had been driven off, and then, with the outside bath temperature raised to 105°, with an oil pump through two liquid air traps at a pressure of about 0.1 mm.   A middle fraction of about 75 cc. was collected and redistilled with the oil pump.   The middle fraction from the second distillation, usually about 60 cc., was then set away in ice-salt to crystallize.   Even when a large quantity of lactic acid is required, it is preferable to carry out the distillation a number of times with small portions because with large quantities greater losses are incurred during the longer heating required at 105°.

In order to minimize anhydride formation in the syrup and in the vapor through overheating, the side arm of the distilling flask, internal diameter about 2 cm., was fused into the neck about 1 cm. above the bulb and was shielded from the heat of the paraffin bath by asbestos board.   For the same reason the filter flask in which the distillate was received was kept at room temperature in a water bath during the collection of the middle fractions.

In about 2 hours the first nuclei of crystals appeared in the second middle fraction set away in ice-salt.   Removed then to the temperature of ice and shaken vigorously, the syrup soon became a solid crystalline mass.   The product at this stage corresponds to that obtained by Krafft and Dÿes.   The degree of anhydro impurity present is shown in Table I.

The crystalline lactic acid was now dissolved in an equal volume of a mixture of equal parts of ethyl and isopropyl ethers (dried over

sodium), complete solution requiring about $\frac{1}{2}$ hour at 37°. After some hours at the temperature of ice-salt, crystallization set in. After another hour, when the contents had become a nearly solid

TABLE I

Titration of Twice Distilled Lactic Acid (Krafft and Dÿes (2))

Weight	First titration, N/14 NaOH	Back titration, N/14 NaOH	Total titration, N/14 NaOH	Calculated value for pure lactic acid, N/14 NaOH	Free lactic acid calculated on basis of sole impurity as	
					Anhydride	Lactide
gm.	*cc.*	*cc.*	*cc.*	*cc.*	*per cent*	*per cent*
1.0447	162 0	1.5	163.5	162 4	93 7	97 2
1.0260	159.2	1 4	160 6	159 5	93 7	97.2
1 0513	163.0	1 5	164 5	163.5	93 7	97 2

TABLE II

Titration of Crystalline Lactic Acid

Specimen	Weight	First titration, N/14 NaOH	Back titration, N/14 NaOH	Total titration, N/14 NaOH	Calculated titration value for pure lactic acid, N/14 NaOH
	gm	*cc.*	*cc.*	*cc.*	*cc.*
l(+)-Lactic acid prepared by recrystallization of distillate three times from the mixed ethers	0 5004	77 84	0 06	77 90	77 86
	0.6836	106 30	0 04	106 34	106 37
	0 7083	110 09	0 04	110.13	110 21
Same after melting and heating to 56° and crystallizing again	0 9963	154 62	0 16	154 78	154 87
	0 7343	114 10	0.28	114 38	114.15
l(+)-Lactic acid from zinc ammonium salt, recrystallized three times from the mixed ethers	0.4182	65 06	0 05	65 11	65.00
	0.4319	67 05	0 05	67 10	67 08
d(−)-Lactic acid from zinc ammonium salt, recrystallized three times from the mixed ethers	0.5498	85.40	0 00	85 40	85 46
	0.3990	61.95	0 00	61 95	62 02

mass of crystals, they were filtered quickly with suction. The crystallization was repeated three times. After the first crystallization, crystallization began in the subsequent ethereal solutions

immediately on cooling under the tap. After the final recrystallization, the fine white crystals were dried at room temperature in a vacuum desiccator. Titration of this product showed that it contained less than 0.1 per cent of impurities such as water, anhydride, or lactide (Table II).

The fractional crystallization from the mixed ethers effects also a separation of the active isomer, which was in excess in the original syrup (here the $l(+)$ form), from the main bulk of inactive material. This was shown by the optical activity of the ethereal mother liquor, which was nearly zero (indicating that no resolution, but only a separation of the excess of active isomer from the inactive form, had occurred), and by the melting point of a mixture of the $l(+)$ acid obtained by this method and some $l(+)$ acid prepared from the zinc ammonium $l(+)$ salt. Separately the melting point of each form was 52.7–52.8°. The mixed melting point was 52.7°. On the other hand, the melting point of an equimolar mixture of the acid obtained by fractional crystallization of the distillate from the crude syrup with that prepared from zinc ammonium $d(-)$-lactate was 16.8° (Table III).

We have not had occasion as yet to try the separation by this method of the $d(-)$ acid from a preponderance of the dl form because all the commercial syrups we secured contained an excess of the $l(+)$ isomer. Nevertheless, we feel confident in recommending the method because the solubility in the mixed ethers of the two active acids prepared from their respective zinc ammonium salts was the same, both being much lower than that of the dl form. dl-Lactic acid can also be crystallized from the mixed ethers, but the temperature of solid CO_2 is necessary for a good yield, while for the active acids 0° is sufficiently low.

The active acids were also prepared by way of their zinc ammonium salts obtained by resolution of the commercial syrup. The method of resolution employed was essentially that described by Purdie and Walker (5). 1000 cc. of lactic acid syrup were made alkaline to litmus with concentrated ammonia, and then boiled until acid again. This was repeated several times until long boiling was necessary to restore a faint acidity. 560 gm. of zinc dl-lactate (air-dried) were now added. The solution was again boiled until all the salt had dissolved, and while still boiling the volume was made up to 2 liters. It was filtered rapidly with suction while

still hot, and then transferred to a clean and dry 4 liter beaker. The beaker was placed in a bath of ice water and stirred. When cool it was seeded with about 0.1 gm. of finely powdered zinc ammonium $l(+)$-lactate. Within a short time after the seed had been thoroughly stirred in, the crystals of the optically active zinc ammonium salt began to cloud the solution. The process was controlled by microscopical examination of the crystals from time to time. The optically active double salt forms short, relatively wide, single, rectangular or square prisms; the inactive salt forms long narrow rods frequently arranged in radiating clusters. The inactive form appeared when the solution was too dilute, or when it was set away at 0–2°. When the syrup was too concentrated, crystallization was very slow, and after 24 hours only a poor yield of active crystals contaminated with the inactive form was obtained. If the solution was too dilute, it was boiled again until a definite quantity of water (determined by weight) was driven off; if too concentrated, it was also boiled, then brought to a definite weight with water, and, after cooling, seeded again. When the conditions were suitable, an abundant quantity of optically active crystals appeared in an hour throughout the solution. The beaker was left to stand overnight at room temperature (15–20°). When microscopical examination showed the copious deposit at the bottom of the beaker to be not more than slightly contaminated with inactive crystals, the supernatant liquor was decanted off and centrifuged. The precipitates in the centrifuge cups and in the beaker were washed three times with 95 per cent alcohol, and, after drying in air, the optical activity of an 8 per cent aqueous solution was measured. The amount of solid remaining undissolved in making the 8 per cent solution was a good measure of the degree of contamination with inactive salt.

After the first batch of active crystals had been separated from the syrup, there was dissolved in it a quantity of zinc dl-lactate, equal to the weight of the active double salt obtained, and then it was seeded with the opposite active form. Purdie and Walker and others have reported obtaining large quantities of both active forms by this method of repeated crystallization after seeding alternately with one and then the other active form. In spite of many attempts this method always failed us. Repeatedly on seeding with zinc ammonium $d(-)$-lactate we obtained either the dl

form, or large quantities of the $l(+)$ double salt.   From one batch of syrup in three successive crystallizations we obtained over a kilo of the $l(+)$ double salt, although the last two seedings were with $d(-)$ double salt.   The probable reason for our failure is that the syrup contained too great an excess of the $l(+)$ isomer.   We did succeed in obtaining a small quantity of the $d(-)$ double salt each time on seeding a virgin syrup with $d(-)$ seed, although it contained an excess of the $l(+)$ form.

We found also that when zinc ammonium $d(-)$-lactate was mixed with a large quantity of the double salt of the dl acid, the active salt could be separated out by warming to 55° for about an hour and then setting aside at room temperature overnight.   We did not succeed in obtaining the double salt of the $l(+)$ acid by this method, probably because of the excess of the $d(-)$ in the crude syrup.   This experience suggests that large yields of either form of active zinc ammonium lactate (3 to 4 times that separated by Purdie and Walker) can be obtained by their method of seeding a supersaturated solution if the initial lactic acid syrup contains an excess of the same active form as the seed added.   The opposite form can also be obtained by seeding a virgin syrup, as in our case, but the yields are small.   If the crude syrup contains a 20 per cent or greater excess of one active form, the preferable method for obtaining a large quantity of the pure isomer, which is in excess, is distillation and crystallization from the mixed ethers as described above, without previous precipitation of the active zinc ammonium salt.

The $l(+)$ seed was prepared as follows: A quantity of zinc $l(+)$-lactate was converted to the ammonium salt by treatment with H_2S, followed by ammonia, and was then added to twice the equivalent quantity of lactate in the form of the zinc salt.   The combined solutions were concentrated on the water bath to a syrup, and then cooled.   The short rectangular prisms of the active double salt obtained were separated from the syrup by suction filtration, washed with 95 per cent alcohol, and air-dried.   The $d(-)$ seed was obtained by means of the morphine salt by Patterson and Forsyth's modification (6) of the method of Irvine (7).

The following was the typical method of preparation of the active acid from its double salt.   500 gm. of zinc ammonium $d(-)$-lactate were dissolved in 1200 cc. of cold water and filtered imme-

diately. The clear filtrate was set away overnight at about 2°. The next morning the precipitate of zinc lactate was separated by suction filtration and washed with distilled water until the washings no longer gave a positive test with Nessler's reagent. The salt was air-dried to constant weight and its water of crystallization determined by heating at 100° to constant weight. The theoretical value for the optically active form is 12.9 per cent, corresponding to 2 molecules of water of crystallization. The *dl* form contains 3 molecules of water of crystallization. When the water of crystallization was greater than 12.9 per cent, the salt was recrystallized until the theoretical value was obtained. If the zinc salt was pure from the outset, the collected filtrate and washings of ammonium lactate were boiled with $Ca(OH)_2$ to drive off the ammonia, and the free acid was obtained by precipitating the calcium with oxalic or sulfuric acid. If the zinc salt was impure, this free acid was converted to the zinc salt by boiling with zinc oxide, and then recrystallized until the theoretical percentage of water of crystallization was obtained. The free acid was liberated from the zinc salts by treatment with H_2S.[2] The combined aqueous solutions of the free acid were now concentrated at 60° with a water pump to a syrup, then distilled, and crystallized from the mixture of ethyl and isopropyl ethers as described above. In this manner about 120 gm. of free acid were obtained from about 500 gm. of each active double salt.

The purity of the free acids prepared, *i.e.* their freedom from anhydride, lactide, and water, was determined by titration with $N/14$ NaOH and phenolphthalein as indicator. The following standardized technique was employed. Between 0.4 and 1.0 gm. of the acid was transferred quickly to a tared weighing bottle, which was then covered, weighed, and inserted with tongs into a wide necked flask containing 100 cc. of CO_2-free distilled water.

[2] Free lactic acid is adsorbed in large quantities by such precipitates as zinc sulfide, calcium sulfate, and calcium oxalate, and boiling several times with large quantities of distilled water was necessary in order to avoid large losses. The precipitates were washed until the washings gave a negative reaction in the following test described by Denigès (8). 0.2 cc. of solution and 2 cc. of concentrated H_2SO_4 are heated in a boiling water bath for 2 minutes. After cooling under the tap, a drop of an alcoholic solution of guaiacol is added. A fuchsin red color develops with 0.01 mg. of lactic acid.

The stopper was then shaken off the weighing bottle, and the titration begun with a stream of CO_2-free nitrogen bubbling continuously through the solution. When the apparent end-point was reached, the weighing bottle and lid were removed with tongs and rinsed with CO_2-free water, the rinsings being collected in the flask containing the lactic acid. The solution was now brought to a boil and again titrated to the first appearance of pink. The total alkali added to this point was designated as the first titration value. 5 cc. of N/14 NaOH were now added and the solution again boiled for 3 minutes, after which the remaining excess of alkali was back titrated with N/14 HCl. The addition of excess alkali, boiling, and back titration were repeated until the difference between the acid used and the alkali added in one back titration was not more than 0.2 cc. The difference between the total excess alkali added and the amount of N/14 HCl used in the back titration was noted as the back titration value. If lactide and anhydride are present (in the absence of a significant amount of water), the sum of the initial titration figure and the total acid liberated by boiling in excess of alkali is greater than the titration figure calculated on the assumption that the lactic acid weighed out was free of water, anhydride, or lactide. If T cc. of N/14 NaOH is the total titration figure observed per gm. of material weighed out, the percentage of free lactic acid is given by the formula $\dfrac{172.7 - T}{17.3} \times 100$, on the assumption that the only impurity is anhydride, and by the formula $\dfrac{194.4 - T}{39} \times 100$ if the impurity is assumed to be solely lactide. Typical titration results with pure and impure lactic acids are given in Tables I and II.

Table III summarizes the melting points, dissociation constants, and hygroscopic properties of the pure acids. The melting point determinations were made on 10 to 12 gm. samples in a wide test-tube stirred constantly with dry nitrogen. The inside temperature was read with an Anschütz thermometer. The temperature of the water bath outside was kept about 1° higher than the inside during the melting, and 1° lower during the subsequent crystallization. The temperatures are the equilibrium temperatures observed during the melting when both phases were present. The crystallization temperatures were 1° lower. The melting points

of the pure active forms observed are much higher than the values of 25–27° given in Landolt-Börnstein (3) or the "International critical tables" (4), which are based on the work of Jungfleisch and Godchot (9). The low values found by these authors are probably to be attributed to incomplete resolution. This surmise is supported not only by the difference in the melting points, but also by the failure of other workers (7) and ourselves to obtain a satisfactory resolution by the method described by Jungfleisch and Godchot. The melting point of *dl*-lactic acid prepared by melting together and then crystallizing equal quantities of the

TABLE III

Summary of Some of the Properties of the Optically Active Lactic Acids

Specimen	Melting point	Water absorbed from the air at room temperature; fraction of original weight	Dissociation constant at 25°
	°C.		pK
d(−)-Lactic acid from recrystallization of distillate (Specimen A)	52 7	0 in 4 hrs.; 2% in 20 hrs.	3 81 ± 0.01
d(−)-Lactic acid from zinc ammonium salt (Specimen B)	52 8	0 in 4 hrs.; 2% in 20 hrs.	3.83 ± 0.01
2 parts Specimen A + 1 part Specimen B	52 7		
l(+)-Lactic acid from zinc ammonium salt	52 8	1% in 4 hrs.; 3% in 20 hrs.	3 79 ± 0 01
Equal weights of Specimen A and *l*(+)-lactic acid	16 8	In liquid state 3% in 3 hrs.; 10% in 15 hrs.	3 81 ± 0 01

pure *l*(+) and *d*(−) acids was found to be 16.8°, nearly 1° lower than the melting point given by Krafft and Dÿes for their *dl*-lactic acid. The probable explanation for this difference is that, apart from contamination with water or anhydro compounds, the product obtained by Krafft and Dÿes was a mixture of the *dl* and one of the active forms. Such mixtures we have found melt at temperatures higher than 16.8°, according to the degree of excess of one of the active forms. The general experience has been that commercial lactic acid syrup, from which Krafft and Dÿes obtained their product, contains nearly always an excess of one of

the active forms; and this mixed composition is carried over into the distillates.

The dissociation constants were determined in the usual manner by electrometric titration with Moloney hydrogen electrodes (10) in duplicate, against a saturated calomel half-cell. In the computation of the dissociation constants we have assumed the activity coefficient of the undissociated acid to be 1, which is justified by the freezing point data given in the "International critical tables." The activity of the lactate ions was estimated by the simplified form of the Debye-Hückel equation. The values obtained, there-

TABLE IV

Optical Rotations of Lactic Acid and Its Salts When $\lambda = 5461$ Å., *at 21-22°*

	Concentration	α	$[\alpha]_{Hg}^{21-22}$	$[M]_{Hg}^{21-22}$
	gm. per cent	*degrees*		
$d(-)$-Lactic acid	8 00	+0 41	+2.6	+2.3
" "	4.00	+0.18	+2.3	+2.1
$l(+)$-Lactic "	8 00	−0.41	−2.6	−2.3
" "	4 00	−0.16	−2.0	−1 8
Zinc ammonium $l(+)$-lactate	8.00	−1.10	−6.9	−8.9
" " $d(-)$-lactate	8 00	+1.10	+6.9	+8 9
Sodium $l(+)$-lactate	4.20	−1.15	−13.7	−15.8
" $d(-)$-lactate	7.05	+1 70	+12.1	+13.5

fore, approximate thermodynamic dissociation constants, pK (as distinguished from titration constants, usually designated as pK').

The avidity of the free acids for water was determined approximately by exposing a weighed quantity of the acid to the air at room temperature in an open weighing bottle. Table III shows that the pure optically active acids are only slightly hygroscopic. This also runs counter to the description usually given, which is derived from the papers of Jungfleisch and Godchot. As in the case of the difference in the melting points, the discrepancy probably is to be ascribed to incomplete resolution, and possibly also to contamination of the product obtained by these authors by water and anhydride, since the free acids were prepared from their

quinine salts by the distillation method of Krafft and Dÿes, and were not purified further.

The pure optically active acids are relatively quite stable. Kept in a desiccator at room temperature, both forms remained unchanged for more than a month; after 6 months, only a small amount of anhydride formation was found to have occurred. The second group of figures in Table II shows that only a small amount of anhydride formation occurs during the melting of the crystals.

The rotations of the free acids and their salts are given in Table IV. The measurements were made at 21- 22° in a 2 dm. tube, with the mercury green line (λ 5461 Å.). The difference in the specific rotations of the two sodium salts is the usual effect of changing the concentration.

The biological activity of the two optically active forms was tested with lactic acid dehydrogenase prepared from muscle by the method of von Szent-Györgyi (11). When the $l(+)$ form was added to the enzyme and methylene blue in an evacuated Thunberg vessel, the dye was quickly decolorized. The $d(-)$ form, on the other hand, was quite inactive, giving a longer decoloration time than the enzyme alone. We are indebted to the kindness of Mr. H. F. Schott for this examination of the two forms of lactic acid prepared, and for his active interest and many helpful suggestions throughout the course of this work.

<div align="center">SUMMARY</div>

1. Two methods are described for obtaining optically active lactic acid (both isomers) from a commercial aqueous syrup.

2. A method is described of preparing the active isomers in a crystalline state, free of water, anhydride, and lactide.

3. Some of the properties of the crystalline acids are described. The following physical constants are given: melting points, $l(+)$-lactic acid, 52.8°; $d(-)$-lactic acid, 52.8°; dl-lactic acid, 16.8°; the acid dissociation constant of the three forms at 25° is pK = 3.81 ± 0.02.

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ARGININE METABOLISM

II. THE RELATION OF THE ARGININE CONTENT OF THE DIET TO THE CREATINE-CREATININE PRODUCTION DURING GROWTH

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In a former communication from this laboratory (Scull and Rose, 1930) evidence was presented for the fact that during the growth of young rats upon an arginine-low diet, the increments of tissue arginine are 2 to 3 times as large as may be accounted for by the arginine intake. These data were interpreted as indicating that arginine may be synthesized, and at least in the species employed is not an indispensable dietary component.

At the same time that the above results were secured, and upon the same animals, another aspect of arginine metabolism was investigated, namely, the relation of the intake of the amino acid to creatine-creatinine production. After the latter experiments had been completed (Scull and Rose, unpublished data) it was observed that the yeast extract employed as a source of vitamin B factors contained a chromogenic material which responded to the Jaffe creatinine test. Despite the fact that the substance behaved atypically as compared with creatinine, inasmuch as the orange-red color largely faded on dilution of the solution, it was feared that the apparent creatinine values in the urines or tissues might have been too high. The data, therefore, were discarded. Recently the experiments have been repeated with the use of a much more satisfactory yeast extract than that formerly employed.

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* The experimental data in this paper are taken from a thesis submitted by Curtis E. Meyer in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Physiological Chemistry in the Graduate School of the University of Illinois.

Furthermore, corrections have been introduced for the slight chromogenic action of the new yeast concentrate. With these modifications the results confirm the earlier findings. However, it is to the new data only that attention is directed in this paper.

In view of the synthesis of arginine (Scull and Rose, 1930), one would scarcely expect increases in its intake to lead to alterations in creatine-creatinine formation. Furthermore, the remarkable uniformity in the output of creatinine in a given individual upon a diet devoid of creatine and creatinine, and the relative constancy in the proportions of creatine in the tissues despite wide qualitative and quantitative variations in food consumption, appear to indicate quite strongly that creatinine results from a special type of *endogenous* change, and that creatine is synthesized by the organism only in so far as it is required as an essential tissue constituent. Nevertheless, the literature records numerous attempts to associate the formation of creatine and creatinine with the consumption of arginine. Doubtless the fact that the latter is the only known protein component which contains the guanidine group characteristic of creatine is largely responsible for this attempted correlation. Most of the papers dealing with this aspect of metabolism are critically discussed in the excellent monograph of Hunter (1928), and in the recent review of Rose (1933) Hunter points out that the majority of such studies have failed entirely to provide evidence for a direct transformation of exogenous arginine into creatine or creatinine; and those which have furnished what appear to be positive results are capable of some other interpretation, such as a non-specific stimulation in metabolism by the amino acid in question. He states (p. 227): "So large a body of almost purely negative evidence leads one rather forcibly to suspect that, if creatine is related to arginine at all, its mother substance must be not the free amino acid, but the still combined arginine of the muscle or other protein." A similar conclusion was expressed in 1918 by Rose, Dimmitt, and Bartlett when they said (p. 612): "the synthesis of creatine is either an anabolic reaction limited quantitatively to the needs of the body for creatine, or is a catabolic reaction associated with the disintegration of tissue protein alone."

Nothing has transpired since 1918 to lead us to alter our position in this matter. Indeed, investigations conducted in this labora-

tory and elsewhere have served to confirm our original opinion. Thus Hyde and Rose (1929) administered arginine to two human subjects for 6 and 8 weeks respectively. The subjects, a young man and a young woman, received daily doses of the amino acid equivalent to 1 gm. of creatine. Despite the large intakes, representing in the two individuals a possible excess production of creatine amounting to 42 and 56 gm. respectively, no evidence of an increased excretion of either creatine or creatinine was secured. These results are in striking contrast to the observations made by Rose, Ellis, and Helming (1928) upon the same female following the administration of corresponding daily doses of creatine. In the latter experiment, 6.0 gm. of extra creatine and 14.6 gm. of extra creatinine were excreted in the course of 7 weeks. Thus the dissimilarity in the physiological behavior of arginine and creatine provides additional evidence against the alleged direct transformation of the former into the latter. Data of similar import were secured by Grant, Christman, and Lewis (1929) in experiments upon dogs. These authors state (p. 231) that the "oral administration of arginine for a period of 35 days failed to influence the excretion of urinary creatine or creatinine, although exogenous creatine in small amounts resulted in prompt increases in both these catabolites."

The results of the above investigations demonstrate, we believe, that the production of urinary creatine and creatinine is not augmented by an increased intake of exogenous arginine. Nor is the creatine content of the entire animal altered by the administration of diets varying widely in the proportions of combined arginine. Thus Chanutin (1930) has shown that rats receiving gelatin or edestin (high in arginine) have no greater content of creatine than do animals upon diets of casein (relatively low in arginine). But while the administration of excessive quantities of the amino acid fails to promote or stimulate creatine-creatinine formation, the possibility of limiting the production of these metabolites by *curtailing* the arginine intake *below the normal level* deserves further consideration (*cf.* Rose, pp. 198–199, 1933). From this point of view also, the dispensable nature of the amino acid would seem to negate the idea that a decreased supply would inhibit creatine-creatinine production. Even if creatine is anabolized, in so far as it is required by the tissues, through the inter-

mediate stage of arginine, the synthesis of the latter would be expected to compensate for the diminished intake in the food. However, the possibility that arginine synthesis might not be sufficiently rapid or extensive to meet the exigencies of growth, involving the production of both arginine-containing proteins and possibly creatine, led us to conduct the experiments outlined below.

EXPERIMENTAL

The methods were similar to those employed in Paper I (Scull and Rose, 1930), and involved a comparison of the arginine intake of growing rats receiving an arginine-low diet, and the creatine-creatinine formation, in order to determine whether the latter can be accounted for by the amount of the amino acid consumed in the basal ration and vitamin supplement.   The creatine-creatinine production was measured by determining for each animal the increment in tissue creatine (creatine plus creatinine) incidental to growth, and the output of total creatinine in the urine.   At the beginning of the experiments, litter mates of the animals employed in the growth studies were killed and subjected to analysis *in toto* for total creatinine.   The other members of each litter were killed and analyzed after they had received the experimental diet for a period of 53 days.   In the meantime they had gained 77 to 110 gm. each.

The reader is referred to the paper of Scull and Rose (1930) for the method used in preparing the arginine-low amino acid mixture, which served as the source of nitrogen in the ration, and for a description of the procedure employed in determining the arginine content of the food materials.   In analyzing the yeast extract for arginine, 25 gm. were refluxed for 10 hours on a sand bath with 120 cc. of sulfuric acid (30 cc. of concentrated sulfuric acid and 90 cc. of distilled water).   The solution was then diluted to 1500 cc., and the sulfate ions were approximately removed by the addition of powdered barium hydroxide.   The barium sulfate precipitate was filtered off, suspended in 1500 cc. of water, heated with steam, and filtered.   This process was repeated three additional times to insure complete removal of the hydrolytic products.   The original filtrate and washings were then combined, and the last traces of sulfuric acid were quantitatively removed by the cautious addition of barium hydroxide solution.   After again filtering, the solution

was concentrated *in vacuo* to about 150 cc., transferred to a 200 cc. volumetric flask, and diluted to the mark with distilled water. Aliquots were taken for the determination of arginine.   In Table I are recorded the results of the analyses of the arginine-low amino acid mixture and the hydrolyzed yeast extract with and without added arginine.   The figures are in remarkably close agreement with like data presented by Scull and Rose (1930, p. 114, Table II), and serve to emphasize the reliability of the analytical procedure.

TABLE I

Arginine Content of Amino Acid Mixture and Yeast Extract

Material	Weight of sample	Arginine added	Dixanthydrylurea	Arginine		Recovery	Arginine content
				Theory	Found*		
	mg	*mg.*	*mg*	*mg.*	*mg*	*per cent*	*per cent*
Arginine-low	5058 0	0	49 2		20 4		0.38
amino acid	4135.0	0	39 6		16 4		0 39
mixture	3034 5	0	27 6		11 4		0.37
	5290 7	34 8	134 4	54 9	55 6	101 2	(0.39)
	4005 6	34 8	123.6	50 0	51 2	102 4	(0 41)
	3020 2	34 8	112 5	46 3	46 6	100 6	(0.39)
Yeast ex-	1000	0	62.7		26 0		2.60
tract (Har-	1000	0	65 8		27 3		2 73
ris)	1500	0	97.4		40.4		2.68
	2000	0	133 9		55 5		2.77
	1000	87.0	276.3	113 9	113 5	99 6	(2.65)
	1500	52 2	221 3	92.5	91 7	99 1	(2 63)
	2000	34 8	213 8	88 6	88 6	100 0	(2.69)

The figures in parentheses represent the percentage of arginine in the samples after deducting the added arginine from the arginine found.

* Dixanthydrylurea $\times$ 0.4143 = arginine.

The urines were collected by supporting the cages, in which the animals were housed, over large glass funnels which emptied into bottles containing toluene.   In the apex of each funnel a small strainer retained particles of food and fecal material which otherwise would have entered the receiving vessels.   Twice daily the funnels were washed down with a fine stream of water.   The samples were preserved under toluene until the end of the experiment.   The combined urine and washings from each animal were then diluted to 2000 cc., and subjected to analysis for total creati-

nine according to the procedure of Folin (1914), 5 cc. samples being employed, and the color compared with a standard containing 0.75 mg. of creatinine.

The total creatinine content of each rat was estimated by the use of a slight modification of the method of Rose, Helmer, and Chanutin (1927).   For this purpose, after the removal of the alimentary contents, as described in Paper I (Scull and Rose, 1930), the entire animal was thoroughly ground and mixed.   10 gm. portions of the mixed tissues were introduced into 300 cc. flasks, and each was treated with 200 cc. of 2 N sulfuric acid.   The flasks were then covered with lead foil, and autoclaved for 45 minutes at 15 pounds pressure.   After cooling, each solution was transferred quantitatively to a 500 cc. volumetric flask, and treated with 180 cc. of 2 N sodium hydroxide and 50 cc. of 10 per cent sodium tungstate $(Na_2WO_4 \cdot 2H_2O)$.   The contents were then diluted to the mark, thoroughly mixed, and allowed to stand for about 2 hours before filtering.   10 cc. samples of the filtrate were introduced into centrifuge tubes, treated with 5 cc. portions of freshly prepared alkaline picrate solution as in the Folin-Wu (1919) procedure, and mixed by inversion.   At the same time, 20 cc. of a standard creatinine solution were placed in a small flask, and treated with 10 cc. of the alkaline picrate solution.   The unknown was then immediately centrifuged, and the clear supernatant fluid was compared in a colorimeter against the standard.

The modifications of the original procedure of Rose, Helmer, and Chanutin were occasioned by the fact that in the present investigation the entire animal, instead of the muscle tissue alone, was subjected to analysis.   In order to insure a uniform sample, 10 gm. instead of 1 gm. portions were autoclaved.   The longer standing of the hydrolyzed tissue solutions preliminary to filtration, and the centrifugation after the treatment of the samples with alkaline picrate, were necessary because of the development of a turbidity on the addition of sodium hydroxide due to the presence of calcium and magnesium salts derived from the skeletal structures.   With the changes indicated the method yields reliable results on the mixed tissues.   It should be noted, however, that the colorimetric readings, as in the original procedure, should be completed within 12 to 15 minutes from the time the color is developed.

The composition of the diet is shown in Table II.   In addition

to the basal ration, which was fed *ad libitum*, each animal received 200 mg. of yeast extract daily in the form of two pills fed at approximately 12 hour intervals. Thus during the 53 days of the experiments each rat ingested 10.6 gm. of the yeast extract. Inasmuch as the latter contained a small amount of material which responded to the Jaffe test for creatinine, several colorimetric determinations of the chromogenic substance were carried out. These yielded values ranging from 0.56 mg. to 0.60 mg., expressed as creatinine, per gm. of yeast extract. If one makes the improba-

TABLE II

*Composition of Diet**

	gm
Arginine-low amino acid mixture†	11.1
Cystine	0.3
Tryptophane	0 2
Histidine..	0 4
Dextrin....	43 0
Sucrose.....	15 0
Lard.......	19 0
Cod liver oil....	5 0
Salt mixture‡..	4 0
Agar........	2 0
	100 0

* Vitamin B factors were supplied in the form of two pills daily each containing 100 mg. of Harris yeast extract, and administered at approximately 12 hour intervals.

† *Cf.* Scull and Rose (1930).

‡ Osborne and Mendel (1919).

ble assumption that the chromogenic agent of yeast is actually creatinine,[1] and on the basis of the highest value obtained calculates the intake from this source, it is evident that 6.36 mg. might have been secured by each animal during the 53 day period. In order to allow for this remote possibility we have subtracted 7 mg. of creatinine from the output of each rat before calculating the arginine equivalent of the urinary creatinine.

[1] Several attempts were made in this laboratory by Mr. Paul Halmbacher and the senior author to isolate creatinine from large quantities of hydrolyzed and unhydrolyzed yeast and yeast extract. All such efforts were unsuccessful.

TABLE III
Creatine Content of Tissues

Litter	Rat No.[*] and sex	Initial body weight		Final body weight		Total creatine-creatinine content[†]				Creatine increment[†]
		Live	Minus alimentary contents	Live	Minus alimentary contents	Initial	Final	Initial	Final	
		gm.	*gm.*	*gm.*	*gm.*	*mg. per cent*	*mg. per cent*	*mg.*	*mg.*	*mg.*
A	1♂	62	59			182				
	2♂	62	58			182				
	3♀	56	52			182				
	4♀	56	52			182				
	5♀	63	56			181				
	1921♂	59	55	142	138	182	219	100	302	202
	1922♂	60	56	139	136	182	222	102	302	200
	1923♀	61	57	148	144	182	225	104	324	220
	1924♀	63	59	150	145	182	223	107	323	216
	1925♀	62	58	147	143	182	220	106	315	209
B	6♂	80	74			185				
	7♂	70	63			183				
	8♂	70	64			183				
	9♀	66	60			184				
	1932♂	72	66	154	150	184	217	121	326	205
	1933♂	66	60	143	139	184	222	110	309	199
	1934♀	66	60	152	147	184	217	110	319	209
	1935♀	69	63	157	153	184	217	114	332	218
C	10♂	62	59			181				
	11♀	47	43			175				
	12♀	52	50			178				
	13♀	52	50			176				
	14♀	56	52			182				
	1936♂	56	53	152	147	178	217	94	319	225
	1937♀	54	51	142	138	178	220	91	304	213
	1938♀	53	50	149	145	178	220	89	319	230
	1939♀	54	51	146	142	178	215	91	305	214
	1940♀	49	46	159	153	178	221	82	338	256

* Rats 1 to 14 inclusive are the controls which were killed and analyzed at the beginning of the experiment.

† Expressed as creatine (not creatinine).

The results of the experiments are summarized in Tables III to V inclusive.   In Table III are presented the data on the creatine content of the tissues.   Rats 1 to 14 inclusive were the controls

which were analyzed at the beginning of the experiments.   The
animals which were permitted to grow are numbered 1921 to 1925
and 1932 to 1940 inclusive.   The average weight of the alimentary
contents and the average percentage of creatine in the controls
of each litter were assumed to represent the corresponding values
in the litter mates which received the diet.   The figures in the
last column of Table III, representing the increments in tissue
creatine, demonstrate that each of the fourteen animals increased
its content of this component by a value ranging from 199 to 256
mg.   Furthermore, the increases are very closely proportional to
the total gains in body weight.

TABLE IV
Output of Total Creatinine

Rat No. and sex	Total increase in body weight	Urinary creatinine*	Rat No. and sex	Total increase in body weight	Urinary creatinine*
	gm.	*mg.*		*mg.*	*mg.*
1921 ♂	83	271	1934 ♀	86	262
1922 ♂	79	266	1935 ♀	88	293
1923 ♀	87	273	1936 ♂	96	267
1924 ♀	87	262	1937 ♀	88	292
1925 ♀	85	281	1938 ♀	96	289
1932 ♂	82	295	1939 ♀	92	299
1933 ♂	77	290	1940 ♀	110	280

* Creatine + creatinine expressed as creatine.   Each of these values is
reduced by 7 mg., representing the maximum apparent creatinine content
of the yeast extract consumed by the animal during the 53 day experiment.

In Table IV are recorded the figures representing the output of
total creatinine for the 53 day period.   As will be observed, these
values range from 262 to 299 mg. after correction is made for the
apparent creatinine content of the yeast extract ingested by the
animals.

In Table V are compared the arginine consumption and the
creatine-creatinine production of each rat.   *In calculating the
arginine intakes we employed the highest figures secured in the analyses
of the amino acid mixture, and of the yeast extract (Table I), namely
0.41 and 2.77 per cent respectively.   Thus the figures in the fifth
column of Table V represent maximum values.*

The data are self-explanatory. They demonstrate that the intakes of arginine were much too small to account for the creatine-creatinine stored in the tissues and excreted in the urines.   Indeed, the "arginine deficits" shown in the last column of Table V range from 248 to 316 mg.   If one takes into consideration the arginine used in the formation of tissue proteins (Scull and Rose, 1930) the deficits become enormously greater.   These results appear to

TABLE V

A Comparison of Arginine Intake and Creatine-Creatinine Production

Rat No. and sex	Arginine equivalent to increment in tissue creatine	Arginine equivalent to output of total creatinine	Total arginine required for creatine-creatinine formation	Arginine intake*	"Arginine deficit"
	mg.	*mg*	*mg.*	*mg.*	*mg.*
1921 ♂	268	417	685	436	249
1922 ♂	266	410	676	443	233
1923 ♀	292	420	712	457	255
1924 ♀	287	403	690	442	248
1925 ♀	278	433	711	455	256
1932 ♂	271	454	725	447	278
1933 ♂	264	447	711	436	275
1934 ♀	278	403	681	452	229
1935 ♀	290	451	741	447	294
1936 ♂	299	411	710	447	263
1937 ♀	283	450	733	449	284
1938 ♀	306	445	751	461	290
1939 ♀	284	460	744	454	290
1940 ♀	340	431	771	455	316

* Each animal received 294 mg. of arginine from the yeast extract. The balance in each case was derived from the arginine-low amino acid mixture.

dispose of the idea that the synthesis of creatine and creatinine is dependent upon the intake of *preformed* arginine.   On the other hand, the data do not exclude the possibility that these metabolites may be manufactured *through arginine as an intermediate*, inasmuch as this amino acid can be synthesized by the rat.

SUMMARY

Comparative studies have been made of the arginine intake of rats upon an arginine-low diet, and the production of creatine

and creatinine as measured by the increment in tissue creatine incidental to growth and the output of total creatinine in the urine. The results show that under the conditions employed, the formation of total creatinine is very much greater than may be accounted for by the arginine ingestion, even when the use of arginine in the synthesis of tissue proteins is omitted from consideration. Evidently, the production of creatine and creatinine is not dependent upon the quantity of preformed arginine in the food.

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THE ACTION OF SULFHYDRYL COMPOUNDS ON INSULIN*

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It has been repeatedly shown that the physiological activity of insulin is irreversibly destroyed by the action of various reducing agents such as zinc and hydrochloric acid, activated magnesium, sodium amalgam, hydrogen sulfide (1–3). The nature of the reacting groups in the insulin molecule has not been ascertained; it is, however, a reasonable assumption that the disulfide linkages of the cystine, which is a quantitatively important constituent of insulin, are the first to be attacked. On the other hand, it has been observed in some of the reactions mentioned above, that small but definite amounts of ammonia are liberated during the period of inactivation, pointing to the participation of groups other than the disulfide linkages. Du Vigneaud, Fitch, Pekarek, and Lockwood (4) have recently investigated the reaction between insulin and thiol compounds such as cysteine and glutathione, mild reducing agents with a presumably higher degree of specificity for disulfide linkages than nascent hydrogen. They observed that in neutral or slightly alkaline solution rapid inactivation took place, giving rise to the appearance of free sulfhydryl groups in the insulin as manifested by a positive nitroprusside test. This result was interpreted as proof of the importance of the disulfide bonds in the insulin to its physiological action. The claim that the reduction of the disulfide bonds is the primary and probably sole reaction occurring is sustained by experiments of Hopkins (5) on the interaction of oxidized glutathione with denatured

serum or muscle proteins, and of Mirsky and Anson (6) on the determination of preformed sulfhydryl groups in proteins. From the work of these authors it appears that the protein disulfide groups are completely reduced by an excess of a soluble thiol compound, and that the sulfhydryl groups thus formed in the protein can be reoxidized by an excess of a soluble disulfide acting on them. The extent of reduction corresponds to that of the subsequent reoxidation and thus indicates that in the protein only disulfide and sulfhydryl linkages respectively are involved in these reactions.

The aim of the present investigation was to establish the degree of correlation between the reduction of disulfide in insulin by cysteine and the destruction of the physiological activity. The apparently greater specificity of this reaction as compared with other inactivating processes rendered a quantitative inquiry of this kind justifiable and feasible. The method consisted in following the time curve of inactivation in solutions buffered to pH 7.2 and determining the simultaneous decrease in cysteine concentration by iodometric titration.

Another point investigated was the possible participation of the so called alkali-labile group in this type of inactivation. When insulin is inactivated with dilute alkali, some ammonia is split off from the molecule. As first shown by Freudenberg, Dirscherl, and Eyer (1), the amount of ammonia liberated, if certain mild conditions of alkali treatment are maintained, increases with time and finally reaches a maximum value when the inactivation is complete. In different preparations this limiting value is approximately proportional to the physiological activity. This fact led Freudenberg and his collaborators to consider this alkali-labile group, from which the ammonia is derived, as a structure particularly important to the physiological action. According to their view this group is conceived as a part of the hypothetical active group. As mentioned above, the alkali-labile group is attacked in the inactivation of insulin by some metallic reductants such as sodium amalgam or magnesium (2). The amount of ammonia liberated is, however, only half of that obtained on alkaline inactivation; the other half can be made available by subsequent treatment with dilute alkali. It seemed of interest to inquire whether similar results would be obtained in the mild and rapidly progressing reduction of insulin with sulfhydryl compounds. In the case

of a positive finding the alkali-sensitive group could then be considered, with some degree of probability, as associated with a cystine group.

Iodometric Determination of Cysteine—It has been pointed out by several investigators that the direct iodometric titration of small amounts of cysteine and other sulfhydryl compounds is beset with certain difficulties. The amount of iodine reduced depends on the acidity, temperature, and most important of all, on the concentration of the cysteine solution, as has been noted by Okuda (7) and others. In general, too high values are obtained, since the oxidation is carried beyond the disulfide state, probably leading to the formation of the sulfinic (8) and sulfonic acids (9). In attempting to develop a reliable method for the present purpose we observed that nearly theoretical titration values, corresponding to the reaction, $2R—SH + I_2 \rightarrow R—S—S—R + 2HI$, could be obtained by working at a fairly high degree of acidity and in the presence of an excess of potassium iodide. This principle has been previously used by Perlzweig and Delrue (10) and others for the determination of glutathione, and has recently been studied, in the case of cysteine, by Lucas and King (11). In the present work, which was completed after the paper of the latter authors appeared, similar conditions in regard to acidity and KI concentration were maintained. The titrations were, however, performed at room temperature, which accounts for the fact that figures slightly higher than the theoretical were obtained. For the problem on hand it was less important to attain absolute stoichiometric precision than good reproducibility. Satisfactory results were obtained at temperatures between 18–22° with a final concentration in HCl of about 0.25 N, and 1 per cent of KI. Higher acidity does not diminish the accuracy, but a higher content in KI obscures the end-point when starch is employed as an indicator. The final volume of the titrated solution seldom exceeded 8 cc. The iodine solution (0.01 N) should be added at a slow rate, about 2 drops per second. We found it advantageous to avoid reaction of the cysteine with a considerable excess of iodine, but preferred to add the iodine solution to the first appearance of a blue coloration, and to titrate back with 0.01 N thiosulfate after 1 minute's standing, if

the blue color had persisted over that period.   Then 0.1 cc. more of iodine solution was added, the excess again titrated back after 1 minute, and this process repeated, till no more iodine was taken up.   Usually the second and third titrations agreed within 0.01 cc.   In this way a large excess of iodine is avoided in the final stage of the titration.

In ten titrations of 2 cc. of a solution containing 4.0 mg. of cysteine hydrochloride, to which 0.25 cc. of 20 per cent KI and 0.25 cc. of 5.7 N HCl were added, the mean value obtained was 2.354 ± 0.011 cc. of 0.01 N I; largest deviation from mean, 0.017 cc.; theory, 2.280 cc. of 0.01 N I.

Determination of Cysteine after Reaction with Insulin—Insulin was allowed to react with cysteine at pH 7.2 for the time specified, the mixture was then acidified by addition of trichloroacetic acid, and the cysteine remaining unoxidized was determined in the filtrate.   Oxygen was rigidly excluded during the period of interaction, as it was observed that the autoxidizability of cysteine was considerably increased by the presence of insulin.   A few preliminary experiments to investigate this point were performed in a Barcroft-Warburg apparatus, the results of which are compiled in Fig. 1.   The O_2 uptake in air may be increased over the normal rate of autoxidation 2 to 3 times, depending on the insulin preparation used.   It may be noted that an analogous observation was made by Rosenthal and Voegtlin (12) on the autoxidation of glutathione in the presence of egg albumin.

For the reduction experiment a vessel consisting of two chambers of 30 and 5 cc. capacity respectively was used, the contents of which could be mixed by tilting the vessel.   Both chambers were provided with glass stoppers, one of which served to establish connection with a gas inlet tube sealed into the female part of the ground joint.   The larger chamber contained the insulin dissolved in the hydrochloric acid-cysteine solution, while into the smaller side chamber the NaOH and buffer for neutralization were introduced. All solutions were added from standardized microburettes.   After the solution had been placed in both chambers, the vessel was weighed on an analytical balance, evacuated to about 20 mm. of Hg, and filled with nitrogen purified by passing through a large combustion tube filled with freshly reduced copper scrapings. This procedure, evacuation and filling with N_2, was repeated four

or five times. Finally the vessel was filled with nitrogen and the contents were mixed by tilting. After the experimental period, trichloroacetic acid was run into the side chamber, while nitrogen was passing out through the open neck into which the burette tip was introduced. After the insulin was precipitated, the nitrogen in the vessel was removed by a current of air, and the vessel again weighed. From both weighings and the known weight of the trichloroacetic acid solution added, the diminution of the total volume of liquid due to evaporation during the evacuation periods
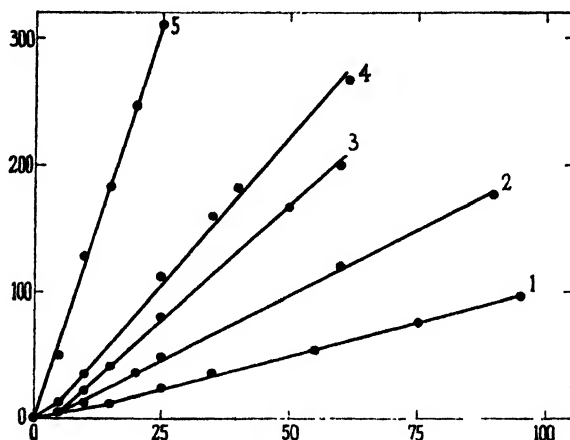
Fig. 1. O_2 uptake of cysteine and cysteine + insulin in air, in phosphate buffer, volume 3 cc., at 20.0°. Curve 1 represents 15 mg. of cysteine-HCl at pH 7.0; Curve 2, 15 mg. of cysteine-HCl at pH 7.29; Curve 3, 15 mg. of cysteine-HCl, 10 mg. of crystalline insulin at pH 7.08; Curve 4, 15 mg. of cysteine-HCl, 10 mg. of crystalline insulin at pH 7.33; Curve 5, 15 mg. of cysteine-HCl, 30 mg. of insulin Preparation B at pH 7.0. The abscissæ represent time in minutes; the ordinates, c.mm. of O_2.

could be calculated. This procedure was preferred to transferring solution and precipitate quantitatively to a volumetric flask since it seemed advisable to keep the dilution of the cysteine at a minimum. After centrifuging off from the precipitated insulin and adding the appropriate amounts of HCl and KI, two aliquots were titrated with 0.01 N I_2 solution. Neutralized cysteine solutions subjected to these operations and allowed to remain up to 18 hours in the vessel filled with nitrogen did not change their titer. Experiments in which casein instead of insulin was used showed

that the danger of losing cysteine by adsorption on the precipitated protein is negligible.

The cysteine employed was obtained from commercial cystine by reduction with tin and hydrochloric acid, and recrystallized twice under nitrogen from 20 per cent HCl. $C_3H_8O_2NSCl + H_2O$: 20.21 per cent Cl; calculated 20.20 per cent.   It was dissolved to the required strength in boiled water and the solution kept under nitrogen in a vessel with a burette attached.   Its cysteine content was controlled each day by titration, the same amounts and conditions being employed as in the insulin experiment.   This was necessary because slight variations of the iodometric equivalent, probably due to temperature differences, were occasionally observed.   All results obtained in the insulin experiments were referred to the average titration value obtained on cysteine alone on the same day.   The calculation of the cysteine oxidized by the insulin was then based on the assumption that the cysteine value, a, obtained on that day represented the amount of cysteine actually employed (10 mg.).   Where b cc. are used up in the titration of the same volume of trichloroacetic acid filtrate after reaction with insulin, it was assumed that $10\, \dfrac{a-b}{a}$ mg. of cysteine had been oxidized in the reaction with insulin.

Example—20.0 mg. of crystalline insulin; 1.00 cc. of cysteine solution = 10 mg. of cysteine-HCl; 0.13 cc. of 0.5 N NaOH; 0.50 cc. of M/30 phosphate buffer (pH 7.2); 0.37 cc. of H_2O, total volume 2.00 cc.; reaction time 25 minutes; 3.00 cc. of 6 per cent trichloroacetic acid solution added.   Water loss by evaporation 0.081 gm.; total volume therefore 4.919 cc.; of this, 2 × 2.0 cc. were titrated, corresponding to 4.07 mg. of cysteine-HCl; 2.241, 2.249 cc. of 0.01 N I reduced; for 4.00 mg. of cysteine, therefore, 2.207, 2.215 cc. of 0.01 N I.   Blank titrations on 4.00 mg. of cysteine-HCl, 2.404, 2.405, 2.401, 2.404 cc. of 0.01 N I; difference of means 0.192 cc.; cysteine-HCl oxidized, 10 × (0.192:2.403) mg. = 0.80 mg.

The amounts of reagents given above were employed in all experiments, the only variables being the period of interaction and the insulin preparations used.   The solutions for the animal experiments were prepared and treated in the same way, and also served for occasional control of the pH.   For injection aliquots were pipetted into the required volume of dilute hydrochloric acid (0.01 N) in order to stop the reaction.   The comparatively high

concentrations of insulin and cysteine were used in the titration experiments because it was originally attempted to check the titration figures by polarimetric measurements. This, however, proved to be not feasible. The results of the titration experiments are recorded in Table I.

Determination of Ammonia after Interaction between Cysteine and Insulin—For the determination of the small amounts of ammonia to be expected in this reaction, the method of Parnas and Heller (13) based on steam distillation under reduced pressure and subsequent Nesslerization was employed. The insulin used, preparation Höchst 27, 12 international units per mg., was allowed to react in 1 per cent solution with an equal amount of cysteine hydrochloride, buffered with borate to pH 8.0, for 30 minutes. After the reaction period an aliquot corresponding to 20 mg. of insulin was brought to pH 9.2 by addition of alkali and N borate buffer of this pH, and distilled. Under the conditions chosen the inactivation is very rapid. After 5 minutes 98 per cent, and after 30 minutes all of the activity was destroyed.

Blank determinations were carried out on insulin and on cysteine under the conditions specified above.

Experiments were also performed to determine the amount of ammonia available by the action of dilute alkali after inactivation with cysteine had taken place. After the reaction period the pH was brought to about 12.5 by addition of an appropriate amount of 0.5 N NaOH (corresponding to the alkalinity of 0.03 N NaOH) and the solution kept at 34° for 24 hours. After this time the liberation of ammonia from the alkali-sensitive group as has been previously shown (1) is complete.

The results of these experiments, including the blank determination, are recorded in Table II. During the interaction with cysteine no ammonia is split off. The small amount liberated is practically the same as in the blank with insulin alone (0.026 per cent) and is presumably derived from ammonium salts contaminating the preparation. In the experiments with subsequent alkali treatment the amount liberated from the insulin alone is much smaller than would be expected from the physiological activity of the preparation used (0.017 per cent, after subtraction of the preformed ammonia). The blank value for cysteine alone is not considerably enhanced by standing in alkaline solution. In the

presence of cysteine, insulin splits off about 3.5 times as much ammonia on treatment with alkali as without cysteine (0.061 and 0.017 per cent respectively).

TABLE 1

Reduction and Inactivation of Insulin by Cysteine

20 mg. of insulin, 10 mg. of cysteine-HCl, in phosphate buffer pH 7.2; volume 2 cc.

Insulin Preparation		Reaction time, min.						
		5	15	30		45	90	300
A	$\dfrac{a-b*}{a}$ cc. 0.01 N I..	0 079	0 114			0 141	0 147	0 153
		2 474	2 474			2 474	2.474	2 474
	Cysteine oxidized, mg....	0 32	0 46			0.57	0 59	0 62
	Total S in insulin reduced, per cent.	22	32			39	41	43
	Residual activity, per cent.	32	0					
B	$\dfrac{a-b}{a}$ cc. 0.01 N I.	0 043	0 111	0 139	0 123		0 155	0 172
		2 358	2 372	2 362	2 372		2 362	2 460
	Cysteine oxidized, mg.	0 18	0 35	0 59,	0 52		0 66	0 70
	Total S in insulin reduced, per cent....	7	13	23	20		25	27
	Residual activity, per cent.	67	43	30			13	2
C	$\dfrac{a-b}{a}$ cc. 0.01 N I ..	0 018	0 062			0 192	0 247	0 213
		2 373	2 391			2 403	2 391	2 373
	Cysteine oxidized, mg.	0 076	0 26			0 80	1 03	0 94
	Total S in insulin reduced, per cent	2	8			23	30	26
	Residual activity, per cent†	55	31	24			16	3

Preparation A—Impure fraction (about 0.8 international unit per mg.) obtained in the purification of commercial insulin by the brucine method. Total S 1.32 per cent; cystine content 3.24, 3.34 per cent (Folin-Marenzi); 2.46 per cent (Sullivan).

Preparation B—Fraction of same commercial insulin, 10 international units per mg.; total S 2.39 per cent, cystine not determined.

Preparation C—Crystalline insulin, 25 international units per mg.; total S 3.1 per cent; cystine content 12.8 per cent (Folin-Looney); 8.2 per cent (Sullivan).

* See text, p. 478; a = mean of four to six titrations, b = mean of two titrations.

† Average of values recorded in Fig. 2.

TABLE II

Ammonia Liberated from Insulin

Treatment		Blank determinations			
		Borate-NaOH	20 mμ cysteine, borate, NaOH	20 mg. insulin, borate, NaOH	20 mg. insulin + 20 mg. cysteine, borate, NaOH
Inactivation with cysteine	NH₃, γ	2 1, 0 min. 3 2, 0 "	3 6, 0 min. 2 9, 0 " 3 3, 90 " 3 5, 90 "	8 2, 30 min. 8 0, 30 "	8 6, 30 min. 7 8, 30 " 7 0, 30 "
	Mean	2 6	3 3	8 1	7 8
	−2 6γ		0 7	5 5	5 2
	NH₃, per cent			0 028	0 026
Inactivation with cysteine followed by treatment with alkali	NH₃, γ		3 6 4 7	9 8 12 2	18.8 24 4
	Mean		4 2	11 5	21 6
	Minus blank from borate-NaOH (2.6γ) " " " borate-NaOH-cysteine (4.2γ)			8 9	17 4
	NH₃ totally liberated, *per cent*			0 045	0 087
	" by action of alkali, *per cent*			0 017	0 061

In the determination of the physiological activity in the various inactivation experiments the rabbit method described by Freudenberg and Dirscherl (14) was employed. In general twelve animals were used for assaying one preparation; in the more
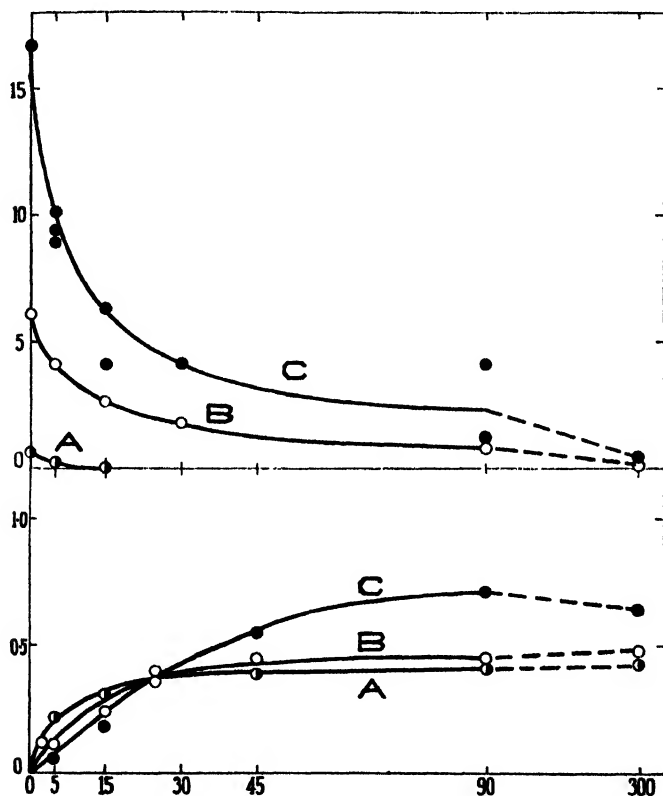
FIG. 2. Inactivation (upper curves) and reduction (lower curves) of insulin by cysteine. The abscissæ show time in minutes. The ordinates of the upper section show physiological activity in laboratory units per mg.; in the lower section, mg. of insulin-cystine reduced. 1 laboratory unit = 1.5 international units. Curves A, B, and C represent Preparations A, B, and C, respectively.

important experiments twenty-four to thirty-six animals. The figures obtained in a series of measurements on partly inactivated solutions were referred to the mean of two to three control assays of the untreated starting material, obtained during the same week.

Oxygen Uptake of Cysteine in Presence of Insulin (Fig. 2)—The measurements were carried out in a Barcroft-Warburg apparatus at 20.0°. In Experiment 3 the cysteine content of the solution was determined afterwards. The figure thus obtained for the cysteine oxidized was somewhat higher than the value calculated from the oxygen uptake, thus indicating that only cysteine had been oxidized.

DISCUSSION

Under the conditions adopted, the reduction of the disulfide linkages in insulin by cysteine is a fairly rapid reaction. The reduction time curves obtained on three insulin preparations are plotted in Fig. 2, together with the corresponding inactivation curves. It can be seen that the reduction approaches a limiting value in all three cases, which is reached more quickly with the less active Preparations A and B, and more slowly with crystalline insulin (Preparation C). The equilibrium value is about the same with Preparation A and Preparation B, but lies on a considerably higher level in the case of Preparation C. No proportionality exists between the maximal reduction attained and the physiological activity of the three preparations. It is therefore clear that the reacting groups cannot be considered as specific for insulin. On the contrary, the disulfide linkages of the contaminating proteins in the impure preparations seem to react somewhat faster under identical conditions than those in crystalline insulin. The ratio of cysteine to protein disulfide naturally determines the rate of reaction. This ratio is undoubtedly lower with the crystalline insulin than with the impure preparations (see below), and this may account for the somewhat slower course of the reaction as shown by Curve C. There is no recognizable correlation between the reduction level and the total sulfur content of the three preparations. Preparation A contained 1.32, Preparation B 2.39, Preparation C 3.10 per cent sulfur; at equilibrium, 43, 27, and 28 per cent of the sulfur, respectively, had reacted. In Preparation A, however, only 65 per cent of the total sulfur was found to be present in form of disulfide groups, as determined by the Folin-Marenzi method, while in crystalline insulin the disulfide groups (not necessarily all in the form of cystine) account for nearly all the sulfur present (15). It must also be remembered, that the Folin-Marenzi method is not

entirely specific for disulfide groups, and that other reducing groups present in protein hydrolysates may also react with the color reagent as has been shown by Sullivan and Hess (16). This makes the high percentage of reduction in Preparation A still more difficult to explain.

In the case of *crystalline insulin* the equilibrium value represents a reduction of somewhat less than one-third of the disulfide linkages, as determined by the Folin-Marenzi method (or of the total sulfur content). The point of time at which this value is reached coincides approximately with the destruction of all of the physiological activity. In the beginning, however, the reduction seems to proceed somewhat more slowly than the inactivation. If one compares the reduction after 5 minutes with the loss of activity at that point of time (49, 49, 42, 34 per cent in four independent experiments) it appears that reduction of only about 5 per cent of the total disulfide present destroys all of the activity of the molecule. We hesitate to lay much emphasis on this conclusion in view of the considerable uncertainty attached to that figure, which represents a difference between the titration values $(a - b)$ very close to the limit of error of the method. Nevertheless it seems to be true—at least in a qualitative sense—that the reduction of a comparatively small number of disulfide linkages suffices for the destruction of the activity.

If one adopts the concept advocated by Freudenberg of a distinct active group installed somewhere in the peptide chains of the insulin protein, this finding would mean that the disulfide linkages in that group are the first to react with the reducing agent. It seems, however, more reasonable to assume that no marked differences exist in the reactivity towards reducing agents of disulfide bonds located in different parts of the molecule; rather that the cleavage of some of these bonds and the concomitant changes in the physicochemical properties of the protein molecule lead to the rapid inactivation observed. An analogous course of events is seen in the action of proteolytic enzymes on insulin (17), in which the inactivation rapidly precedes the splitting of peptide linkages. An explanation involving diminution of molecular size would be more in keeping with the facts in both cases.

No attempt has been made in the present investigation to determine the maximal extent to which the reduction can be carried by a

large excess of cysteine. The value thus obtained would probably be a measure of the total disulfide present in insulin. In order to answer this question the present method, which rests on the determination of comparatively small differences in cysteine concentration, would have to be replaced by an indirect method like that of Mirsky and Anson. The use of a thiol compound such as thioglycollic acid, which offers fewer difficulties in analysis than cysteine, would also seem preferable. Experiments in this direction are in progress.

The reduction of the insulin disulfide by cysteine is apparently not influenced by the presence of cyanide. No inhibition, but rather a slight increase in the rate of the reaction was observed (Table IV). It is therefore unlikely that in this reaction metal complexes play a rôle similar to that observed by Toda (18) in the oxidation of cysteine by methylene blue. The findings, which we were able to confirm, of du Vigneaud and his coworkers that hydrocyanic acid in concentrations not harmful as such to insulin is incapable of inhibiting the inactivation by cysteine, are in keeping with this observation.

An attempt to reactivate insulin solutions inactivated by cysteine by aeration in presence of Fe^{III} in acid or neutral medium, or of dithiodiglycollic acid, was no more successful than du Vigneaud's analogous experiment with oxidized glutathione. Neither does an excess of a soluble disulfide added at the start to the reacting mixture of insulin and the corresponding thiol compound ($1\frac{1}{2}$ mol of dithiodiglycollic acid to 1 mol of thioglycollic acid) inhibit or retard inactivation. All in all, the irreversible nature of the process militates against the idea of an active group comprising only one or two especially reactive disulfide linkages. Such a structure, if capable at all of leading an existence separate from the long "inert" peptide chains, should be susceptible to at least partial reintegration by oxidizing measures. The alternative contention, that the integrity of the whole protein molecule with all its structural details is necessary for exerting the physiological action, is in better agreement with the recognized facts.

Influence of pH—The rate of inactivation increases markedly with rising alkalinity. The range between pH 6 and 8 was investigated with cysteine, thioglycollic acid, and α-thiolactic acid as reducing agents, and the dependence of the reaction on pH was

found to be approximately the same for all three compounds (Table III).   No maximum of the rate of inactivation was observed in that range as in the autoxidation of cysteine (19) and

TABLE III

Influence of pH on Rate of Inactivation of Insulin by —SH

6 mg. of crystalline insulin, 6 mg. of cysteine-HCl·1H$_2$O, or 3.144 mg. of thioglycollic acid, or 3.645 mg. of α-thiolactic acid, 1.5 cc. of phosphate or borate buffer; volume 3.0 cc.; 3 hours under N$_2$ at 20°.

Cysteine		Thioglycollic acid		Thiolactic acid	
pH	Residual activity	pH	Residual activity	pH	Residual activity
	per cent		*per cent*		*per cent*
6.19	92				
6 33	108	6.26	98	6.24	90
6.81	58				
6.98	61	6.86	67	6.80	81
7.19	49	7.19	48		
7.56	26			7 25	35
7.80	2	7 68	14	7.62	18
8 12	2	8.02	0		
8 26	0	8.45	0	8.14	0

TABLE IV

Influence of pH on Rate of Reduction of Insulin by Cysteine

20 mg. of insulin, 10 mg. of cysteine-HCl·1H$_2$O, phosphate or borate buffer, volume 2 cc.

Preparation	Reaction time	pH measured	Cysteine oxidized
	min.		*mg.*
A	15	6.94	0.20
"	15	9.54	0.48
B	90	6.50	0.27
"	90	7.01	0.66
"	90	8.50	1.02
" +0.5 mg. KCN	90	7*	0.77

* Approximate.

glutathione (20), or in the oxidation of glutathione by methylene blue (20).   The reducing value also increases with rising pH (Table IV).

The question whether the alkali-labile group of insulin is involved in this type of inactivation has to be answered in the negative (Table II). The reduction with thiol compounds is therefore more specific than with some of the metallic reductants. By the subsequent action of alkali a small amount of ammonia (0.061 per cent) was split off, 3.7 times as much as in the blank without cysteine. This would indicate that the lability of the reduction product towards alkali is greater than that of the starting material.

SUMMARY

The time course of the reduction by cysteine of the disulfide linkages in three insulin preparations of different physiological strength, and of the simultaneously occurring inactivation, has been followed. No proportionality was observed between maximal reduction and physiological activity. With crystalline insulin the inactivation is complete when the reduction of the disulfide groups reaches a value corresponding to about one-third of the total sulfur present. The time course of both processes in the initial stage, however, makes it probable that reduction of a comparatively small proportion of disulfide groups suffices for the destruction of the physiological activity.

The rate of inactivation of insulin by cysteine increases with rising pH in the range studied (pH 6 to 8). Thioglycollic acid and α-thiolactic acid inactivate insulin over that range with the same velocity as cysteine.

The presence of cyanide inhibits neither inactivation nor reduction of insulin by cysteine.

The alkali-labile group of insulin is not involved in the inactivation by cysteine.

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IMPROVEMENTS IN MANOMETRIC MICRO-KJELDAHL AND BLOOD UREA METHODS

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HYPOBROMITE REAGENT

Since it was introduced by Knopf (1870), the hypobromite reagent for ammonia and urea analyses has been prepared by dissolving bromine in strong alkali solution. The solution thus prepared has three drawbacks: (1) It rapidly weakens on standing, and consequently must be prepared fresh on the same half day in which it is used. The necessity of frequently making up the solution with the inconveniently handled bromine is an annoyance, and the greater part of each solution prepared is likely to be wasted, because it spoils before it can be used. (2) The alkaline hypobromite solution generates oxygen gas slowly, so that the solution becomes supersaturated with oxygen. Unless precautions are taken to get rid of this gas before analyses are made, the oxygen is evolved along with the N_2 from urea or ammonia, and results calculated on the assumption that the total gas is N_2 are too high. (3) When the alkaline hypobromite comes into contact with mercury it forms a precipitate of mercuric bromide which soils the gas apparatus.

Stehle (1920–21) introduced the innovation of preparing two separate solutions, one of NaOH and one of Br in aqueous KBr solution. These were mixed for use in analyses. This procedure obviated the annoyance of the daily preparation of bromine solutions, but was not utilized in the manometric hypobromite micro-Kjeldahl (Van Slyke, 1926–27) and urea (Van Slyke, 1929) methods published from this laboratory, for the reasons that after the

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KBr-Br and NaOH solutions had been mixed they generated oxygen more rapidly than Br-NaOH solutions, and that Stehle's comparatively dilute mixed solution held considerable amounts of air in solution; whereas, the solution of bromine in 40 per cent NaOH adopted for the manometric methods dissolved so little air that the correction for it was slight and without significant temperature variation.

The advantages of both types of hypobromite reagent can, however, be combined by using concentrated instead of dilute KBr and NaOH solutions, so that air solubility is kept low, and by mixing the KBr-Br and NaOH solutions in the gas apparatus for each analysis, so that no time for oxygen formation is permitted. Also, the larger amounts of KBr introduced in the concentrated solution serve to redissolve whatever mercuric bromide is formed in the apparatus and keep the latter continuously clean.

Hypobromite Reagent

Bromine Solution—60 gm. of KBr are dissolved in 100 cc. of water.   2.5 cc. of bromine are dissolved in the KBr solution.   It is convenient to keep a supply of the solution in a dropping bottle, and to measure out the amount for each analysis by drops from the pipette stopper.

40 Per Cent Sodium Hydroxide—40 gm. of NaOH are dissolved in water and brought to 100 cc.

Use of Reagent in Manometric Analyses

For manometric micro-Kjeldahl analyses (Van Slyke, 1926–27) or urea determinations, the solution to be analyzed is first measured into the chamber of the Van Slyke-Neill (1924) apparatus, and is deaerated if necessary.   Then 1.25 cc. of the 40 per cent NaOH are placed in the cup at the top of the chamber, and 0.75 cc. of the bromine solution is added and mixed with the alkali. 1.5 cc. of the mixture are run into the chamber and the analysis is continued in the usual manner.

The chamber must be free from copper salts, which catalyze O_2 formation, from lactic acid, and from caprylic alcohol.

MANOMETRIC BLOOD UREA DETERMINATIONS

One of the writers (Van Slyke, 1929) has described a manometric application of the hypobromite method to the Folin-Wu

(1919) tungstic acid blood filtrate. Because of the considerable amount of non-urea nitrogenous substances in this filtrate which react somewhat with hypobromite, the results showed a positive error averaging 2 mg. of nitrogen per 100 cc. of blood and sometimes reaching a maximum of 4 mg. In consequence the method, despite its ease and speed, could be recommended for use only when, as in non-protein nitrogen determinations, the object is merely to determine whether gross urea retention has occurred, and a maximum possible error of ±2 mg. of urea N per 100 cc. of blood is not important. For the greater accuracy required for comparison of blood urea and urea excretion by the urea clearance, the method did not suffice.

The zinc sulfate precipitant for blood proteins introduced by Somogyi (1929, 1930) for sugar analyses was shown by him (1929) to remove 8 to 15 mg. of non-protein nitrogen, including that of uric acid, glutathione, and ergothionine, per 100 cc. of blood. We have found that the zinc sulfate precipitation eliminates a large part of the non-urea substances which evolve nitrogen when treated with hypobromite, and that it does not remove any of the urea. Use of this filtrate makes the simple hypobromite method sufficiently exact for blood urea clearance determinations. After blood urea analyses were run for some months in this hospital in parallel by both the urease method (Van Slyke, 1929) and the hypobromite method here presented, the latter displaced the urease method for blood analysis in clearance determinations. (For urine analyses the urease method remains the more convenient, because it does not involve preliminary removal of ammonia.)

Reagents

Bromine solution and *40 per cent NaOH* already described.

Acid Zinc Sulfate Solution (Somogyi, 1930)—12.5 gm. of $ZnSO_4 \cdot 7H_2O$ are dissolved in 125 cc. of 0.25 N sulfuric acid and made up to 1 liter with water.

0.75 N Sodium Hydroxide—When 50 cc. of the zinc sulfate solution are titrated with the 0.75 N NaOH, with phenolphthalein as indicator, 6.7 to 6.8 cc. of the alkali should be required. The solution is shaken vigorously during the titration (Somogyi, 1930).

0.62 Per Cent NaCl in Water—This solution has the same solubility for air as the Somogyi blood filtrate.

Procedure

Precipitation of Proteins—1 volume of blood is mixed with 8 volumes of the zinc sulfate solution. The mixture is shaken until it is homogeneous and hemolysis is complete. Then 1 volume of the 0.75 N alkali is added. The mixture is filtered after a few minutes.

Manometric Determination of Urea in Filtrate—The chamber of the manometric apparatus is filled with mercury, of which 1 or 2 cc. are run up into the cup to serve as a seal for the delivery pipette. 5 cc. of the blood filtrate are run from a rubber-tipped pipette through the mercury seal (Van Slyke, 1927, *a*, Fig. 3, p. 125) into the chamber of the apparatus. The mercury in the cup is removed, the chamber is evacuated, and the air is extracted from the filtrate by shaking for 2 minutes. The extracted air is expelled from the chamber without loss of any of the solution. The hypobromite reagent is then added as described in the preceding section of this paper. The mercury in the chamber is lowered to the 50 cc. mark and the chamber is shaken for 1.5 minutes at 25°, 2 minutes at 20°, or 2.5 minutes at 15° to extract the N_2 formed from the urea.

The extracted gas is brought to 0.5 cc. volume for analyses of bloods with ordinary urea content, or to 2 cc. volume if the urea content is so high that the pressure at 0.5 cc. exceeds 400 mm., and the manometer reading p_1 is taken. The gases are then completely expelled, the cock is sealed with mercury, and the solution meniscus is lowered to the same mark at which it stood when the p_1 reading was taken. The p_0 reading, with the chamber gas-free, is then taken.

A blank analysis is performed in which 5 cc. of the 0.62 per cent salt solution replaces the blood filtrate. The c correction of the analysis is the value of $p_1 - p_0$ obtained in the blank analysis. The c correction is due to a slight amount of air, introduced into the chamber, dissolved in the hypobromite solution and a slight amount not completely removed from the blood filtrate by a single extraction. The c correction is about 6 to 8 mm. when the pressure is measured with the gas at 0.5 cc. volume and 1.5 to 2 mm. when it is measured with the gas at 2 cc. volume. The value of c varies so little that it is usually unnecessary to redetermine it

unless the reagents are changed or the room temperature undergoes unusually great variation.

It is not necessary to wash the chamber between analyses.   The slight amount of hypobromite in the film left on the wall after ejection of the solution is reduced by contact with the mercury, so that it has no action on the urea in the next portion of filtrate introduced.

Calculation

The pressure, P_{N_2}, of the nitrogen gas is calculated as

$$P_{N_2} = p_1 - p_0 - c$$

Mg. urea N per 100 cc. blood $= (P_{N_2} \times \text{factor}) - 1.2$

"   "   per 100 cc. blood $= (P_{N_2} \times \text{factor}) - 2.6$

The values of the factors for urea and urea N are given in Table I.   These values are the theoretical ones multiplied by 1.02 to allow for the fact that under the conditions of the blood filtrate analysis 1 mol of urea evolves only 0.98 mol of N_2 gas (Van Slyke, 1929).   When the factors in Table I are used the nitrogen calculated represents the urea, plus a small amount of N_2 evolved from non-urea substances in the blood filtrate.   The data summarized in Table II showed that the average amount of N_2 evolved from non-urea substances in the Somogyi filtrate from human blood is equivalent to 1.2 mg. of urea nitrogen or 2.6 mg. of urea.   Hence subtraction of an empirical correction for this amount is introduced into the calculation.   The correction was found to be practically the same in bloods with marked urea retention as in normal bloods.

In the former paper on the hypobromite method (Van Slyke, 1929) the theoretical factors were used in calculating the urea content of the blood filtrate, and an empirical correction was obtained as in the present case by comparison of hypobromite and urease results.   The present calculation procedure is somewhat more exact.   In the former calculation (Van Slyke, 1929) the use of theoretical factors introduced a 2 per cent negative error in the calculation of the part of the N_2 that was evolved from the urea itself.   As the urea content rose this negative error, in mg. per 100 cc., increased until it finally balanced the positive error due to the

comparatively constant content of non-urea reacting substances in the blood filtrate. The point of balance is reached in the Somogyi filtrates when the urea nitrogen content of the blood is about 60 mg. per 100 cc. and in the Folin-Wu filtrate when it is about 120 mg. If theoretical factors were used to multiply P_{N_2}, therefore, the

TABLE I

Factors by Which P_{N_2} Is Multiplied to Give Urea or Urea Nitrogen per 100 Cc. of Blood

Temperature	Factors giving mg. urea N		Factors giving mg urea	
	$a = 0 5$ cc.	$a = 2$ cc	$a = 0 5$ cc.	$a = 2$ cc
°C.				
15	0.1592	0 636	0 3407	1.363
16	86	34	0 3395	58
17	80	32	80	52
18	75	30	70	48
19	69	27	57	43
20	63	25	45	39
21	58	23	34	34
22	52	21	22	29
23	46	18	11	24
24	41	16	0 3299	20
25	36	14	88	16
26	30	12	77	11
27	25	10	65	05
28	20	08	53	01
29	15	06	42	1 297
30	09	04	30	92
31	04	02	19	87
32	0 1498	00	08	83
33	93	0 598	0 3198	79
34	89	96	87	75

empirical correction should be variable, changing from a negative value for normal bloods to zero when the urea content reached the above point of balance, and to a positive correction for bloods with higher urea content. The error introduced in the former paper (Van Slyke, 1929) by using theoretical factors and a constant correction could, however, in no case exceed 2 per cent of the urea

determined, and would rarely exceed 1 per cent, so that it was within the limits of accuracy of the method.

RESULTS

In the routine comparisons above referred to the hypobromite method was compared with the gasometric urease analysis ("Procedure A, for determinations in a series of blood filtrates," Van Slyke, 1927, *b*, p. 703), devised for rapid routine analyses of series of blood filtrates. This procedure has a maximum error of about 1 mg. of urea N per 100 cc. of blood. In order to obtain a more exact check on the accuracy of the present hypobromite method we have compared it in analyses of nineteen bloods with the gasometric urease procedure applied to 1 cc. samples of whole blood (Van Slyke, 1927, *b*, p. 708), in which the maximum error in bloods of ordinary urea content is about 0.3 mg.[1] For comparison with the former hypobromite procedure on the Folin-Wu precipitate (Van Slyke, 1929), a portion of each blood was also freed of protein by the Folin-Wu tungstic acid method, and the urea in the filtrate was determined by hypobromite. The analyses, both by the urease and the hypobromite methods, were all performed in duplicate.

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[1] In gasometric blood urea determinations by the urease procedure previously published (Van Slyke, 1927, *b*) it is necessary, in order to obtain in 1 minute complete decomposition of the amounts of urea that may be present in uremic bloods, that the urease have a high grade of activity. When the activity is determined by the aeration method of Van Slyke and Cullen (1914), (see also Peters and Van Slyke, 1932, p. 546) the amount of 0.1 N acid neutralized should be at least 40 cc. When the activity is determined by the gasometric method (Van Slyke, 1927, *b*; Peters and Van Slyke, 1932, pp. 377-378) the mg. of urea split per minute per mg. of urease should be at least 0.08 mg. This is 4 times the activity prescribed by Van Slyke and Cullen for use in the aeration urease methods. It is somewhat less, however, than the usual activity of urease prepared by the acetone precipitation method of Van Slyke and Cullen from jack beans. We have found that some commercial jack bean preparations are diluted with inert matter, so that, although sufficiently active for urine analyses, and for gasometric determinations on blood of ordinary urea content, they gave low results with uremic blood. Squibb's Double Strength urease, which is free from such dilution, is adequate for the 1 minute digestions. If urease of half the activity above prescribed is used, the period of digestion in blood analyses must be prolonged to 2 minutes.

The results are given in Table II.

In a previous paper (Van Slyke, 1929) it was shown that the non-urea substances in the Folin-Wu filtrate reacted more slowly than did urea.   Whereas the decomposition of the latter by hypobromite is almost instantaneous, the non-urea substances continued to evolve gas, so that the amounts measured in successive minutes steadily increased.   In the Somogyi filtrate these slowly reacting substances are nearly absent.   For example, in analyses

TABLE II

Comparison of Blood Urea Determinations by Urea and Hypobromite Methods

All data are given in mg. of urea N per 100 cc. of whole blood.

	15 bloods with urea N between 6 and 36			4 bloods with urea N between 70 and 180		
	Urease method on whole blood	Hypobromite method		Urease method on whole blood	Hypobromite method	
		Somogyi filtrate	Folin-Wu filtrate		Somogyi filtrate	Folin-Wu filtrate
Average difference between duplicates...... .. .	0 11	0 08	0 10	0 30	0 23	0 30
Average deviation from urease results...... ..		±0.2*	±0.6*		±1 2*	±0 6*
Maximum deviation from urease results......... ...		±0 5*	±1 2*		±1 4*	±1 1*

* In calculating these results the nitrogen values obtained by multiplying P_{N_2} by the factors in Table I were corrected for non-urea nitrogen by subtracting 1.2 mg. in the case of the Somogyi filtrates and 2.4 mg. in the case of the Folin-Wu filtrates.

of Somogyi and Folin-Wu filtrates from a given blood, the P_{N_2} values measured at 24° after 1.5, 2.5, and 3.5 minutes reaction with hypobromite were 74.0, 76.1, and 77.8 mm. respectively for the Folin-Wu filtrate, and 71.0, 71.8, and 71.9 for the Somogyi filtrate.

SUMMARY

Stehle's device of replacing the usual alkaline hypobromite solution by separate solutions of NaOH and of Br in aqueous KBr

solution, which are mixed for each analysis, has been found not only to give the advantage of a stable bromine solution, but also to eliminate error from spontaneous generation of O_2 which occurs in standing solutions of alkaline hypobromite. The KBr also prevents fouling of the apparatus with mercuric bromide precipitates. This type of hypobromite reagent, in a form adapted to the manometric methods, has proved its convenience and accuracy in routine micro-Kjeldahl and blood urea determinations.

Somogyi's procedure for precipitating the blood proteins with zinc hydroxide is found to remove the greater part of the non-urea substances, present in tungstic acid filtrates, which evolve nitrogen when treated with hypobromite. In bloods with less than 50 mg. of urea nitrogen per 100 cc., use of the Somogyi filtrate decreased the average error of the hypobromite method, checked by careful gasometric urease analyses, to ± 0.2 mg. of urea nitrogen per 100 cc. Use of this filtrate therefore makes the hypobromite method exact enough for blood urea clearances.

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DETERMINATION OF AMMONIA IN BLOOD

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New York)*

In the method here presented Nash and Benedict's (1921) pro-
cedure for aerating the ammonia from blood into an acid-receiving
solution is utilized without modification. For determining the
ammonia obtained, however, we have employed, instead of
Nesslerization, the blue color developed when ammonia is heated
with an alkaline solution of phenol and hypochlorite. This reac-
tion was apparently first utilized for quantitative estimations by
Thomas (1912, 1913), who attributed its discovery to Berthelot
many years before. Thomas found it sensitive to a 1:2,000,000
dilution of ammonia nitrogen, and used it to estimate ammonia
in cerebrospinal fluid. He found that the reaction was also given
by amino acids, but only when they were present in very much
greater concentrations. Orr (1924) used the reaction for direct
determination of ammonia in urine, and Murray (1925) for the
ammonia yielded by blood urea in microanalyses.

We have found that the phenol reagent, when applied as de-
scribed below, is more sensitive than Nessler's solution (Koch
and McMeekin, 1924). A dilution of 0.001 mg. of ammonia
nitrogen in 5 cc. of solution (the minimum obtained in blood
analyses) is just beyond the limit at which Nessler's solution gives
a perceptible color; but with this dilution the phenol reagent still
yields sufficient color for approximate quantitative determination.
Furthermore, the blue product of the phenol reaction behaves like
a true solution, with no tendency to precipitate, while the colored
product obtained as a result of Nessler's reaction is highly insolu-
ble, and its colloidal solution is likely to flocculate in the presence
of traces of caprylic alcohol. This behavior makes Nesslerization

results uncertain if caprylic alcohol is used to prevent foaming of the blood during aeration, since the slight amounts carried over into the receiving solution may suffice to cause the flocculation. The phenol reagent is free from this trouble. Even if caprylic alcohol is emulsified in the blue solution the only effect is a clouding due to suspended droplets of the alcohol, and a reading can be made in the colorimeter, although with less ease than when the solution remains clear. This occasional clouding occurred only in control analyses of standard ammonia solutions, not in blood analyses. The blood seems to hold back the alcohol sufficiently to prevent passage of amounts large enough to cloud the receiving solution.

Reagents

Ammonia-free water, obtained by distilling dilute sulfuric acid and collecting the middle portion of the distillate.

0.1 N hydrochloric acid.

Potassium oxalate and potassium carbonate solution of Nash and Benedict (1921). This contains 15 gm. of oxalate and 10 gm. of anhydrous carbonate in 100 cc. This solution is prepared free of ammonia according to the directions of Folin (1932).

Caprylic alcohol.

Sodium Phenate Reagent—This is made by dissolving 25 gm. of phenol in a small amount of water, adding 50 cc. of 40 per cent sodium hydroxide, and diluting to 100 cc. with ammonia-free water.

Javel water containing 1 gm. of Cl per 100 cc. Dissolve 50 gm. of calcium hypochlorite having 56.5 per cent available Cl in about 500 cc. of hot water. Mix this solution with one containing 50 gm. of anhydrous potassium carbonate in 200 cc. of cold water. Make the whole to a volume of 1 liter. Test the clear solution for excess calcium with K_2CO_3, and add more of the latter if necessary to precipitate all the calcium This reagent should be kept in the ice box in a number of small bottles, so that the chief source of supply is not opened each day, and so that there is a minimum of replacement of solution by air as the reagent is used. The active chlorine is estimated before use and at intervals of a few weeks as follows: To 5 cc. of the Javel water are added 25 cc. of water, 2.5 cc. of 10 per cent potassium iodide, and 2 cc. of glacial

acetic acid.   The mixture is then titrated with 0.1 N thiosulfate which has been freshly standardized against 0.1 N potassium biiodate solution.

Standard Solutions—A stock solution of ammonium sulfate is made by dissolving 0.2358 gm. of ammonium sulfate in water and diluting to 1000 cc.   Each cc. of this solution contains 0.05 mg. of ammonia nitrogen.   From this solution dilutions are made according to Table I.

Potassium oxalate is prepared ammonia-free according to the directions of Folin (1932).   A 30 per cent solution of the neutral salt is prepared and used as an anticoagulant by drying it on the walls of the tube in which the blood is collected for analysis.

TABLE I
Standard Solutions

Volume of stock solution diluted to 100 cc to make standard	Ammonia N in 100 cc dilute standard
cc	mg
6	0 300
5	0 250
4	0 200
3	0 150
2	0 100
1 6	0 080
1 0	0 060
0 8	0 040
0 4	0 020

Procedure

Into the aeration tube described by Nash and Benedict (1921) for the estimation of ammonia in blood measure 5 cc. of blood, 1 cc. of ammonia-free solution of potassium oxalate and potassium carbonate, and 1 drop of caprylic alcohol.   Into the receiving tube measure 5 cc. of ammonia-free water and 3 drops of 0.1 N HCl. Aerate for 10 minutes at the rate of 6 liters of air per minute.   The air, before it reaches the blood, is washed through four wash bottles; the first three contain 5 per cent sulfuric acid and the last one contains 1 per cent hydrochloric acid.

At the end of the aeration period add to the solution in each receiving tube 1 cc. of the sodium phenate solution and 0.5 cc. of

the Javel water.   Mix, place in a boiling water bath for 3 minutes, cool with cold running water to room temperature, and compare the intensity of color with a standard in a colorimeter.

A series of standards, chosen to cover the possible range of ammonia content of the blood, is set up at the same time with each series of analyses.   In each standard tube are placed 5 cc. of a standard solution, 3 drops of 0.1 N hydrochloric acid, and sodium phenate and Javel water as for the blood-receiving tubes.   The standard tubes are heated and cooled as described above.

Calculation

$S/U \times$ mg. ammonia N per 100 cc. of standard used = mg. ammonia N per 100 cc. blood

S = colorimeter reading of standard, which is usually 20 mm.

U =   "   "   " unknown

EXPERIMENTAL

Recovery of Ammonia from Standard Solutions—5 cc. portions of standard solutions were substituted for blood in the aeration apparatus.   The amounts of ammonia recovered are shown in Table II.

Ammonia in Whole Blood—The results obtained from analyses of four samples of the same blood are shown in Table III.   A second 10 minute aeration of the blood begun 1.5 minutes after the end of the first aeration gave less than 0.02 mg. per 100 cc.   A third aeration begun 36 minutes after the end of the second gave 0.02 mg. per 100 cc.   Amounts of ammonia nitrogen of 0.02 mg. per 100 cc. and below give a color reaction that is too pale to read in the colorimeter.   Such values can, however, be approximately measured by comparison with standards in test-tubes viewed against a white background (they are not visible at all with Nessler's solution).

There is evidently a slow formation of ammonia at room temperature in blood plus alkaline oxalate-carbonate solution, but the amount is not sufficient to increase results by more than 0.02 mg. per 100 cc. of blood.   We have tried to reduce this ammonia formation by various devices of other authors, with vacuum distillation as well as aeration, but without success.

The ammonia contents of blood from different vessels determined by the present method are shown in Table IV.

The results were obtained with the assistance of Mr. John

TABLE II

Recovery of Ammonia from Standard Solutions

NH₃-N per 100 cc.

Calculated	Found
mg.	*mg.*
0.030	0.033
0.060	0 059
0 060	0 060
0 100	0 099
0 100	0 098
0 100	0 100
0 200	0 196
0 200	0 197

TABLE III

Ammonia in Whole Blood

NH₃-N per 100 cc blood

Sample No	First aeration	Second aeration after 1 5 min	Third aeration after 36 min.
	mg.	*mg.*	*mg.*
1	0 051	0 02	0 02
2	0 050	0 02	0 02
3	0 041	0 02	0 02
4	0 050	0 02	0 02
Average .	0 048	0 02	0 02

TABLE IV

Ammonia in Whole Blood from Different Blood Vessels

Dog No	Source of blood	NH₃-N per 100 cc	Average
		mg	*mg.*
47	Femoral artery	0.050	0 050
		0 050	
	Jugular vein	0 053	0 051
		0 048	
	Renal vein	0.063	0 064
		0 065	
20	Femoral artery	0.066	0 064
		0.061	
	Jugular vein	0.067	0 066
		0.066	
	Renal vein	0.091	0 095
		0.100	

SUMMARY

Ammonia is removed from blood by the Nash-Benedict aeration procedure, and is determined colorimetrically by Berthelot's reaction with hypochlorite and phenol. This reaction is more sensitive than Nessler's for slight amounts of ammonia, and the colored product is immune to flocculation.

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STUDIES OF GAS AND ELECTROLYTE EQUILIBRIA IN BLOOD

XVII. THE EFFECT OF OXYGENATION AND REDUCTION ON THE CARBON DIOXIDE ABSORPTION CURVE AND THE pK' OF WHOLE BLOOD

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In a previous paper from this laboratory, Van Slyke, Hastings, and Neill (1922) presented data quantitatively describing the effect of change in oxygenation on the CO_2 dissociation curves and buffer values of horse blood.

Recently we have required similar data for dog blood. However, the results of Van Slyke, Hastings, Heidelberger, and Neill (1922), with pure hemoglobin solutions and also those of Hastings, Van Slyke, Neill, Heidelberger, and Harington (1924), have indicated that the buffer values, and also the oxidation-reduction effects on the acidity of the hemoglobin, were quantitatively somewhat different in hemoglobins of the horse and dog. There was accordingly reason to expect similar differences in whole blood from the two species. We have therefore determined the CO_2 dissociation curves and buffer values of oxygenated and reduced dog blood.

The results are here presented, together with data for horse blood recalculated with the aid of blood constants which have been more accurately established since the work on horse blood was published.

EXPERIMENTAL

Technique of Saturation and Analysis

The entire procedure was essentially the same as that used by Van Slyke, Hastings, and Neill (1922) in their Experiments 4, 5, and 6. With the exception of the blood for our Experiment 1, 0.2 per cent potassium oxalate plus 0.1 per cent sodium fluoride was

present in each sample.   In the case of Experiment 1, the blood was obtained after intravenous injection of heparin into the animal.

All samples were saturated with gas mixtures in a water bath at 38°.   The completely oxygenated samples were equilibrated with mixtures of CO_2 and air for 30 minutes.   The reduced samples were subjected to two 20 minute equilibrations with mixtures of CO_2 and hydrogen.   Very nearly complete reduction is indicated in the results given in Tables I to IV.

After separation of the gas and liquid phases, the blood samples were kept immersed in ice until analyzed.

For the blood manometric CO_2 analyses, 2 cc. samples were used. Duplicate results usually agreed to within 0.04 mM per liter.   The oxygen capacities by the technique of Sendroy (1931) were but slightly different from the "total hemoglobin" results by Van Slyke and Hiller's (1929) procedure, in which any methemoglobin present is changed by hyposulfite to reduced hemoglobin, and determined by CO-combining capacity.   The agreement between oxygen capacity and total hemoglobin indicates that no appreciable amounts of methemoglobin were present.

Symbols

The general plan of symbols is that of Van Slyke, Wu, and McLean (1923).   The subscript b indicates whole blood, while s indicates serum or plasma.

$[H_2CO_3]$	= concentration in mM of free physically dissolved CO_2 per liter (part is of course anhydrous CO_2, but for convenience it is all expressed as H_2CO_3)
$[BHCO_3]$	= concentration of combined CO_2 including any that may be combined with Hb as well as with the mineral bases
$[CO_2]$	= concentration of total CO_2 in blood, free and combined
$[p]$	= tension of CO_2 in mm. of mercury
α	= Bunsen solubility coefficient of CO_2, cc. dissolved per cc. of solution
pH_s	= pH of serum or plasma
K'	= apparent first dissociation constant of carbonic acid
pK'	= negative logarithm of K'
$\Delta pK'$	= $pK'_b - pK'_s$, the difference between the values of pK' for whole blood and serum respectively
V_c	= volume of cells in 1 volume of blood
	= ratio $[CO_2]_s/[CO_2]_b$ from Fig. 3 of Van Slyke and Sendroy (1928)

$-\dfrac{d[\text{BHCO}_3]_b}{d[\text{O}_2\text{Hb}]} = \dfrac{\text{decrease in mM bound CO}_2}{\text{increase in mM O}_2 \text{ bound by Hb}}$ at constant pH.

$-\dfrac{d[\text{BHCO}_3]_b}{d\text{pH}_s} =$ buffer value of blood in terms of mM decrease in bound CO_2 per liter per unit pH, increase, Hb oxygenation being constant and the pH changes being caused only by changes in CO_2 tension

$-\dfrac{d[\text{CO}_2]_b}{d[\text{O}_2\text{Hb}]} = \dfrac{\text{decrease in mM total CO}_2}{\text{increase in mM O}_2 \text{ bound by Hb}}$ at constant pH.

$-\dfrac{d[\text{CO}_2]_b}{d\text{pH}_s} =$ decrease in mM total blood CO_2 content per unit increase in pH, oxygenation of Hb being constant and pH, changes being caused only by changes in CO_2 tension

Calculation of CO₂ Absorption Curves—The calculations are made essentially as by Van Slyke, Hastings, and Neill (1922), but the constants used in the equations are derived from later data. The equations employed are the following.

(1) $$[\text{H}_2\text{CO}_3] = \frac{\alpha p}{760 \times 0.02226}$$

(2) $$[\text{BHCO}_3] = [\text{CO}_2] - [\text{H}_2\text{CO}_3]$$

(3) $$\text{pH}_s = \text{pK}'_b + \log \frac{[\text{BHCO}_3]_b}{[\text{H}_2\text{CO}_3]_b} \quad \text{(Hasselbalch's equation)}$$

The values for α are estimated from the data of Van Slyke, Sendroy, Hastings, and Neill (1928), who found α to be 0.51 for serum and 0.454 for cells. For whole blood the value of α (usually about 0.49) is calculated as $0.510 - 0.56\ V_c$. The values for V_c were estimated from the determined oxygen capacities of the bloods by the left-hand scale of Fig. 5.

The value of pK'_b was derived for each specimen of blood from the nomogram of Fig. 5 of the present paper. An approximate value for pH, was first calculated by Equation 3, with 6.15 and 6.165 as the values for pK'_b for reduced and oxygenated specimens, respectively. The approximate pH, thus obtained was used to obtain the value of $\Delta\text{pK}'$ by means of Fig. 5. This value was added to 6.10, the pK'_s value of Hastings, Sendroy, and Van Slyke (1928), to obtain, with Fig. 5, the exact value of pK'_b, which was used in Equation 3 to obtain the exact pH,.

For the recalculations of the experiments of Van Slyke, Hastings, and Neill (1922), their $[\text{CO}_2]_b$ values were multiplied by the factor
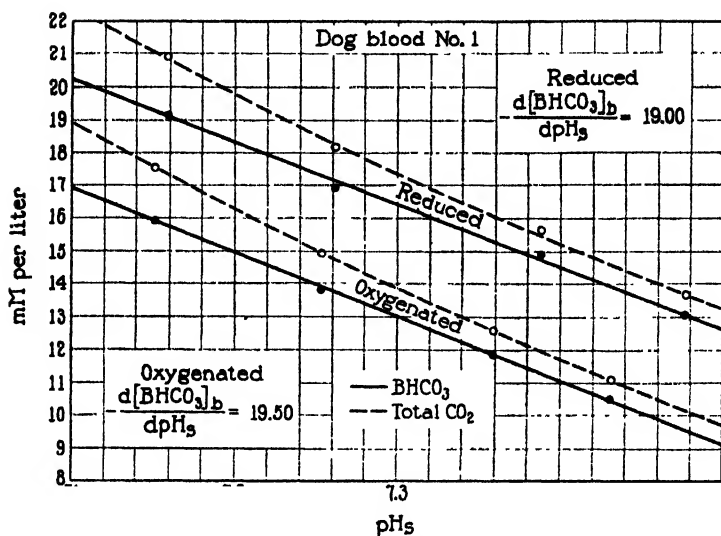
FIG. 1. CO₂ absorption curves for dog Blood 1
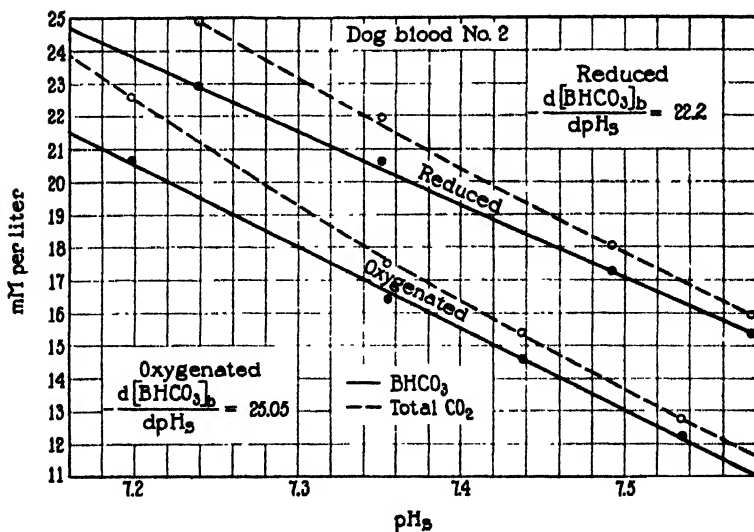
FIG. 2. CO₂ absorption curves for dog Blood 2

1.009 to correct them to the values given by the CO₂ factors of Van Slyke and Sendroy (1927).
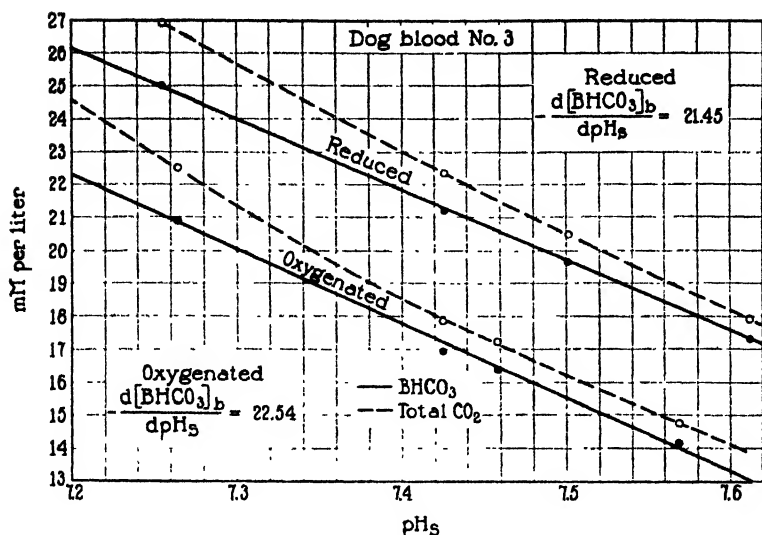
FIG. 3. CO_2 absorption curves for dog Blood 3
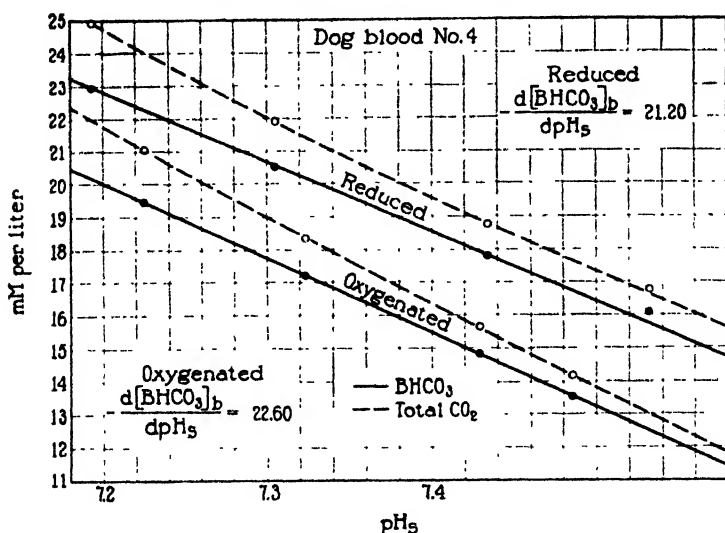
FIG. 4. CO_2 absorption curves for dog Blood 4

CO_2 Absorption Curves of Dog Blood

The experimental data are charted in Figs. 1 to 4. and summarized in Tables I to IV. Table V also contains a summary of results

of the horse blood experiments of Van Slyke, Hastings, and Neill (1922), recalculated by the present method.

The values for dog blood show quantitative differences from those for horse blood. At pH, 7.4, the values of the ratio

TABLE I

Dog Blood 1

Anticoagulant, intravenous heparin; results at 38°; oxygen capacity, 7.22 mM per liter; plasma protein, 5.31 per cent; total hemoglobin, 7.20 mM per liter.

Sample No.	p_{O_2} (approximate)	Total $[O_2]$	$[O_2Hb]$	p_{CO_2}	$[H_2CO_3]_b$	$[CO_2]_b$	$[BHCO_3]_b$	pK'_b	pH,
	mm.	mM per l.	mM per l.	mm.	mM per l	mM per l.	mM per l.		
Reduced									
1	0	0 13	0.13	61.8	1 790	20 92	19 13	6 129	7.159
2	0	0 14	0 14	43.6	1.262	18 20	16 94	6 134	7 261
3	0	0 16	0 16	28 8	0.834	15 63	14 80	6 139	7 388
4	0	0 15	0 15	20 9	0 605	13 67	13 06	6 143	7 477
Oxygenated									
5	146	7 26	7 06	53 8	1 558	17 51	15 95	6 141	7 151
6	146	7.32	7 12	37 2	1 077	14 93	13 85	6 144	7 253
7	146	7 32	7 12	25 3	0 732	12 60	11 87	6 149	7.359
8	146	7.35	7 15	19 2	0 556	11.09	10 53	6 153	7.431

pH,	$[BHCO_3]_b$ Reduced	$[BHCO_3]_b$ Oxygenated	$d[BHCO_3]_b$ Reduced − oxygenated	$-\dfrac{d[BHCO_3]_b}{d[O_2Hb]}$
	mM per l	mM per l.	mM per l.	
7.1	20 23	16 93	3.30	0.474
7.2	18.32	14 97	3 35	0.481
7 3	16.43	13 03	3 40	0 488
7 4	14 53	11.08	3 45	0.495
7 5	12.63	9 13	3 50	0.502

$$\text{Reduced} -\frac{d[BHCO_3]_b}{d\text{pH}_,} = 19\ 00 \qquad \text{Oxygenated} -\frac{d[BHCO_3]_b}{d\text{pH}_,} = 19.50$$

$-\dfrac{d[BHCO_3]_b}{d[O_2Hb]}$ for the dog blood experiments are: 0.495, 0.401, 0.497, and 0.433, average, 0.457; for the horse blood experiments: 0.573, 0.482, and 0.490, average, 0.515. In so far as one

may compare the two sets of experiments, it seems that (a) the variation in this ratio from one experiment to another is similar in blood of both species; (b) the average value of the ratio for dog blood is on a lower level than that for horse blood.   The latter

TABLE II

Dog Blood 2

Anticoagulant, $K_2C_2O_4$ + NaF; results at 38°; oxygen capacity, 10.00 mM per liter; total hemoglobin, 10.03 mM per liter.

Sample No.	PO_2 (approximate)	Total [O_2]	[O_2Hb]	PCO_2	[H_2CO_3]	[CO_2]b	[$BHCO_3$]b	pK_b'	pH_s
	mm	mM per l.	mM per l.	mm	mM per l.	mM per l	mM per l.		
Reduced									
1	0	0 07	0 07	65 4	1 866	24 86	22.99	6.148	7.239
2	0	0 05	0 05	45 8	1.307	21 94	20 63	6 153	7 351
3	0	0 05	0 05	28 2	0 804	18 08	17.28	6 160	7.492
4	0	0 04	0 04	20 9	0 596	15 98	15.38	6 166	7 578
Oxygenated									
5	148	9 82	9 62	66 5	1 897	22 58	20 68	6.160	7 198
6	148	9 87	9 67	37.7	1 075	17 52	16 44	6 169	7 355
7	148	9.84	9 64	28 1	0 802	15 42	14 62	6 177	7 438
8	148	9 86	9 66	19 2	0 548	12 79	12 24	6 186	7 535

pH_s	[$BHCO_3$]b Reduced	[$BHCO_3$]b Oxygenated	d[$BHCO_3$]b Reduced − oxygenated	$-\dfrac{d[BHCO_3]_b}{d[O_2Hb]}$
	mM per l.	mM per l.	mM per l.	
7 2	23.82	20 52	3 30	0.344
7 3	21.58	18 02	3 56	0.371
7 4	19.37	15 52	3 85	0.401
7 5	17 07	13 02	4.05	0.422

$$\text{Reduced} -\frac{d[BHCO_3]_b}{dpH_s} = 22.33 \quad \text{Oxygenated} -\frac{d[BHCO_3]_b}{dpH_s} = 25.05$$

inference is in harmony with the work of Van Slyke, Hastings, Heidelberger, and Neill (1922), and also with that of Hastings, Van Slyke, Neill, Heidelberger, and Harington (1924).   The latter authors found at pH 7.4 for similar solutions of recrystallized dog

and horse hemoglobin, values of $\dfrac{d[\text{BHb}]}{d[\text{O}_2\text{Hb}]}$ of 0.60 and 0.66, respectively, $d[\text{BHb}]$ being calculated as $-d[\text{BHCO}_3]$.

TABLE III
Dog Blood 3

Anticoagulant, $K_2C_2O_4$ + NaF; results at 38°; oxygen capacity, 8.78 mM per liter; plasma protein, 4.40 per cent; total hemoglobin, 8.94 mM per liter.

Sample No.	p_{O_2} (pprox-imate)	Total [O_2]	[O_2Hb]	p_{CO_2}	[H_2CO_3]$_b$	[CO_2]$_b$	[BHCO$_3$]$_b$	pK'$_b$	pH$_s$
	mm.	*m M per l.*	*m M per l*	*m m*	*m M per l.*	*m M per l*	*m M per l*		
Reduced									
1	0	0 11	0 11	67 1	1 926	26 92	24 99	6 142	7 255
2	0	0 04	0 04	39 0	1 120	22 33	21 21	6 148	7 426
3	0	0.03	0 03	30 7	0 882	20 51	19 63	6 154	7 501
4	0	0 04	0 04	21 3	0 612	17 93	17.32	6 160	7 612
Oxygenated									
5	149	8 64	8 44	56 8	1 631	22 50	20 87	6 157	7 264
6	149	8 79	8 59	32 4	0 930	17 87	16 94	6 165	7 425
7	149	8 71	8 51	29 3	0 842	17 24	16.40	6 168	7 458
8	149	8 67	8 47	20 0	0 574	14.74	14 17	6 175	7 568

pH$_s$	[BHCO$_3$]$_b$ Reduced	[BHCO$_3$]$_b$ Oxygenated	$d[\text{BHCO}_3]_b$ Reduced − oxygenated	$-\dfrac{d[\text{BHCO}_3]_b}{d[\text{O}_2\text{Hb}]}$
	m M per l	*m M per l.*	*m M per l.*	
7.2	26.14	22.30	3 84	0 455
7.3	24 00	20 04	3 96	0 468
7 4	21 84	17 77	4 07	0 482
7.5	19 72	15 52	4 20	0.497
7.6	17 57	13.28	4 29	0.508

Reduced $-\dfrac{d[\text{BHCO}_3]_b}{d\text{pH}_s} = 21.45$ Oxygenated $-\dfrac{d[\text{BHCO}_3]_b}{d\text{pH}_s} = 22.54$

Transformation of d[BHCO₃] into d[CO₂] Values

Values for $\dfrac{d[\text{BHCO}_3]}{d[\text{O}_2\text{Hb}]}$ and $\dfrac{d[\text{BHCO}_3]}{d[\text{pH}_s]}$ may be transformed into corresponding values of the increment of total CO_2 content, $d[\text{CO}_2]$, or *vice versa*, by applying the formula

(4) $\qquad d[\text{CO}_2] = (1 + 10^{\text{pK}' - \text{pH}})\,(d[\text{BHCO}_3])$

From Equation 6, Table IV, of Austin *et al.* (1922) it is evident that $[CO_2] = (1 + 10^{pK' - pH})$ $[BHCO_3]$. From the general rule of calculus, that $d(ax) = adx$, it follows that $d[CO_2] = (1 + 10^{pK' - pH})$ $(d[BHCO_3])$. This, however, only holds for a constant value of the factor $(1 + 10^{pK' - pH})$; *viz.*, for a constant pH.

<div align="center">

TABLE IV

Dog Blood 4

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Anticoagulant, $K_2C_2O_4$ + NaF; results at 38°; oxygen capacity, 7.34 mM per liter; plasma protein, 4.50 per cent; total hemoglobin, 7.35 mM per liter.

Sample No.	pO_2 (approximate)	Total $[O_2]$	$[O_2Hb]$	pCO_2	$[H_2CO_3]_b$	$[CO_2]_b$	$[BHCO_3]_b$	pK'_b	pH_s
	mm.	mM per l.	mM per l.	mm.	mM per l	mM per l	mM per l		
Reduced									
1	0	0 05	0 05	69 0	1 997	24 94	22 94	6 132	7 193
2	0	0 04	0 04	48 0	1 389	21 91	20 52	6 135	7.305
3	0	0 05	0 05	31 4	0 909	18 74	17 83	6 141	7 434
4	0	0 07	0 07	22 8	0 660	16 76	16 10	6 145	7 532
Oxygenated									
5	150	7.11	6 91	56 0	1 621	21 10	19 48	6 145	7.225
6	150	7 23	7 03	39 9	1 155	18 38	17 22	6 150	7.323
7	150	7 23	7 03	27 2	0 788	15 63	14 84	6 154	7 429
8	150	7 17	6 97	22 0	0 637	14 17	13.53	6 158	7.485

pH_s	$[BHCO_3]_b$ Reduced	$[BHCO_3]_b$ Oxygenated	$d[BHCO_3]_b$ Reduced − oxygenated	$-\dfrac{d[BHCO_3]_b}{d[O_2Hb]}$
	mM per l.	mM per l.	mM per l	
7 2	22.82	20 03	2 79	0 391
7 3	20 68	17.74	2 94	0.412
7 4	18 56	15.47	3 09	0.433
7 5	16 45	13 20	3 20	0 448

Reduced $-\dfrac{d[BHCO_3]_b}{dpH_s} = 21\ 20$ Oxygenated $-\dfrac{d[BHCO_3]_b}{dpH_s} = 22.60$

Example—If the buffer value of a given blood at $pH_s = 7.4$ is expressible in mM terms as $-\dfrac{d[BHCO_3]}{dpH_s} = 20$, and pK' for whole blood is 6.16, it follows that $-\dfrac{d[CO_2]}{dpH_s} = (1 + 10^{6.16 - 7.40}) \times 20$ $= (1 + 10^{-1.24}) \times 20 = 1.058 \times 20 = 21.16$.

Construction of Nomogram of Fig. 5

When Equation 3 is used with values of BHCO$_3$ and H$_2$CO$_3$ obtained from analysis of serum or plasma, Hastings, Sendroy, and Van Slyke (1928) have found that pK'$_s$ has a nearly constant value of 6.10.   When, however, the equation is used with BHCO$_3$ and H$_2$CO$_3$ values obtained from analysis of whole blood, a higher pK' value must be used to give the correct pH$_s$, because the ratio BHCO$_3$:H$_2$CO$_3$ is lower in cells, and therefore in whole blood, than in plasma.   The greater the cell content the greater will be the difference, ΔpK' = pK'$_b$ − pK'$_s$.   Furthermore in a given blood

TABLE V

Recalculated Results of Horse Blood Experiments of Van Slyke, Hastings, and Neill (1922)

Blood No ... Anticoagulant..	4 K$_2$C$_2$O$_4$ + NaF	5 K$_2$C$_2$O$_4$ + NaF	6 Defibrinated
Reduced − $\dfrac{d[\text{BHCO}_3]_b}{d\text{pH}_s}$	22 23	21 12	23.12
Oxygenated − $\dfrac{d[\text{BHCO}_3]_b}{d\text{pH}_s}$.	23 63	22 10	24.35
− $\dfrac{d[\text{BHCO}_3]_b}{d[\text{O}_2\text{Hb}]}$ at pH$_s$			
7.1	0 516	0 437	0 439
7.2	0 531	0 452	0 458
7.3	0 551	0 470	0 476
7 4	0 573	0 482	0 490
7 5	0 587	0.495	0.511

the value of ΔpK' increases with pH$_s$, for the reason that the difference in BHCO$_3$ concentration between cells and serum increases with pH$_s$, over physiological ranges.   These factors have been treated theoretically and quantitatively by Van Slyke, Wu, and McLean (1923), who also calculated the values of ΔpK' in oxygenated blood with varying hemoglobin content and pH$_s$. Their calculations were based on the difference in base-binding power between the plasma and cell colloids and the theoretically calculated resultant differences in HCO$_3^-$ concentrations between cells and plasma.   Their ΔpK' values agreed, within the limits of experimental error, with values empirically determined by War-

burg (1922) and Peters, Bulger, and Eisenman (1923–24). Van Slyke, Hastings, Murray, and Sendroy (1925) determined the effect of oxygenation and reduction of hemoglobin on the HCO_3^-
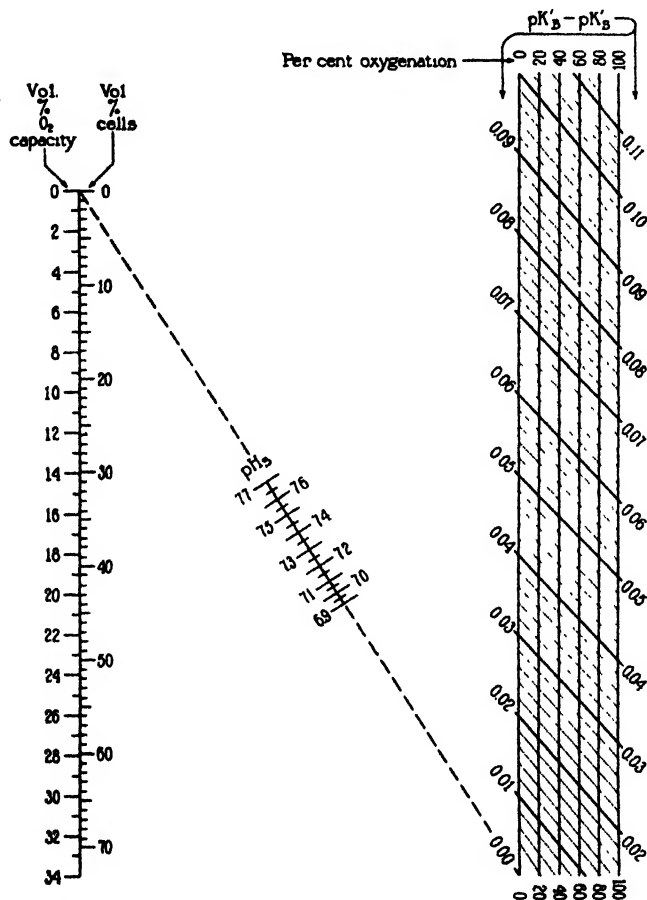
FIG. 5. Line chart for pK′ value for whole blood as a function of hemoglobin content, oxygenation, and pH$_s$. A straight line through the left-hand and pH$_s$ scales cuts the set of scales on the right at a point indicating the value of ΔpK′ which is added to pK′$_s$ to give pK′$_b$.

distribution in blood, and, calculating their data by the theoretical procedure of Van Slyke, Wu, and McLean, prepared a nomogram in which ΔpK′ could be graphically estimated for either oxygenated or reduced blood of any ordinary hemoglobin content or pH$_s$.

Since this nomogram was published, however, more precise data on the constants for the solubility of CO_2 in cells and serum have been obtained (Van Slyke, Sendroy, Hastings, and Neill, 1928), and the distribution ratio of CO_2 between cells and serum at varying pH, and hemoglobin oxygenation has been experimentally determined with increased exactness (Van Slyke and Sendroy, 1928). It appears desirable to prepare a new nomogram based on these more precise data. It also appears desirable to have the nomogram so constructed that it can be used for direct application of the analytically determined CO_2 values in terms of concentrations per unit volume of whole blood, in place of the values in terms of CO_2 per kilo of water present in the blood, which, for reasons of advantage in theoretical treatment, were used by Van Slyke, Wu, and McLean. We have accordingly prepared Fig. 5 by the following mode of construction.

From Van Slyke, Wu, and McLean's Equation 38, we have:

$$(5) \qquad \frac{K'_s}{K'_b} = \frac{[H_2CO_3]_b}{[H_2CO_3]_s} \times \frac{[BHCO_3]_s}{[BHCO_3]_b}$$

In order to transform this equation into one in terms of CO_2 concentrations, CO_2 tensions, and solubility coefficients, we make the following substitutions.

For $[H_2CO_3]_s$ we substitute $0.0591 \, \alpha_s p$.

For $[H_2CO_3]_b$ we substitute $0.0591 \alpha_b p$.

The value of the serum solubility coefficient, α_s, was determined by Van Slyke and Sendroy (1928) to be 0.510. For cells they found an average $\alpha_c = 0.454$. For the whole blood, containing V_c volume of cells per unit volume of blood, the coefficient is calculated as $\alpha_b = 0.510 - 0.56 \, V_c$. Graphically α_b for blood of any cell content can be interpolated in Fig. 6 of Van Slyke, Sendroy, Hastings, and Neill (1928) on a straight line drawn from an ordinate for $\alpha = 0.510$ for $V_c = 0$ to the ordinate representing $\alpha = 0.454$ for $V_c = 1$.

For $[BHCO_3]_s$ we substitute $[CO_2]_s - 0.0591 \, \alpha_s p$ (Equation 2, Peters and Van Slyke, 1931, p. 881, Table 57).

For $[CO_2]_s$ in this expression we then substitute $0.0591 \, \alpha_s p$ $(10^{pH_s - pK'_s} + 1)$ (Equation 5, Peters and Van Slyke, Table 57). For pK'_s we substitute 6.10.

For $[BHCO_3]_b$ we substitute similarly $[CO_2]_b - 0.0591 \, \alpha_b p$.

For $[CO_2]_b$ in this expression, however, we substitute $[CO_2]_s/f$, where f is the ratio, $[CO_2]_s/[CO_2]_b$, found by Van Slyke and Sendroy (1928).   With these substitutions we obtain

$$\frac{K'_s}{K'_b} = \frac{0.0591\ \alpha_b p}{0.0591\ \alpha_s p} \times \frac{0.0591\ \alpha_s p\ (10^{pH_s - 6\,1} + 1) - 0.0591\ \alpha_s p}{1/f \times 0.0591\ \alpha_s p\ (10^{pH_s - 6\,1} + 1) - 0.0591\ \alpha_s p}$$

Simplification of the above gives:

(6)
$$\frac{K'_s}{K'_b} = \frac{\alpha_b}{\alpha_s} \times \frac{10^{pH_s - 6\,10}}{\dfrac{1}{f}\ (10^{pH_s - 6\,10} + 1) - \dfrac{\alpha_b}{\alpha_s}}$$

$$\frac{\alpha_b}{\alpha_s} = \frac{0.510 - 0.56\ V_c}{0.510}$$

(7)
$$\Delta pK' = \log \frac{K'_s}{K'_b} = pK'_b - pK'_s.$$

By means of Equations 6 and 7 a set of curves was prepared, as in Fig. 6,a of Van Slyke, Wu, and McLean, relating, for blood of 10, 20, etc. volumes per cent of cells, the value of pK' to pH$_s$ in oxygenated blood.   A similar set of curves was prepared for reduced blood.   With data from these curves the nomogram in Fig. 5 was prepared by empirical methods similar to those used by Van Slyke and Sendroy (1928).   The nomogram, since it is empirical, is not absolutely exact.   Except at the extremities of the scales, however, the values it gives for pK' do not differ by more than 0.003 from the values calculated by Equation 6.

The O_2 capacities on the left-hand scale are plotted on the assumption of the mean normal relationship between cell volume and blood hemoglobin content, expressed by the formula, $100\ V_c = 2.15 \times$ (volume per cent of O_2 capacity) (Van Slyke and Sendroy, 1928).

When pK'$_b$ values estimated with Fig. 5 are used in calculating pH$_s$ by the Hasselbalch-Henderson equation (Equation 3), the value of α_b used in calculating $[H_2CO_3]_b$ (by Equation 1) *must be the same α_b value that has been used in computing Fig. 5, viz.* 0.510 − 0.56 V_c.   As shown on pp. 796–797 of Van Slyke, Sendroy, Hastings, and Neill's (1928) paper, an error of several per cent in α does not significantly affect the pH calculated by the

Hasselbalch-Henderson equation, *so long as the same α is used in computing pK' and in the subsequent calculations by Equation 3 with this pK'.*

SUMMARY

1. The effect of oxygenation and reduction on the bicarbonate content and buffer value of dog blood has been studied.

2. Previous data on horse blood have been recalculated with the more precise constants now available.

3. At pH 7.4 the increase in combined CO$_2$ caused by removing 1 mM of O$_2$ has been found to average 0.52 mM for horse blood and 0.45 mM for dog blood.   The values for the two species differ to approximately the same degree as the values 0.66 and 0.60 previously found for solutions of the crystalline hemoglobins of the two respective species at the same pH.

4. The buffer value of dog blood is also slightly less, per unit of hemoglobin content, than the buffer value of horse blood.   The difference between whole bloods of the two species is again similar to that between solutions of the crystallized hemoglobins.

5. The Hasselbalch pK' values for whole blood of varying hemoglobin content and oxygenation have been recalculated and expressed in a line chart.

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THE HEAT PRECIPITATION OF INSULIN*

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While attempting to hydrolyze crystalline insulin under very
mild conditions du Vigneaud, Geiling, and Eddy (1) observed that
a solution of insulin in 0.1 N HCl when heated in a boiling water
bath yielded a flocculent precipitate which came down rather
slowly, reaching complete precipitation in 1½ to 2 hours. Al-
though this acid-insoluble material exerted no physiological action
when injected in suspension, reactivation to an active acid-soluble
form could be brought about by dissolving the precipitate in very
dilute alkali followed by immediate acidification. In a later in-
vestigation it was found that this regeneration of activity could be
effected by dissolving the dilute acid-insoluble material in cold 20
per cent HCl (2). Either method of regeneration gave a product
similar in general properties to the original insulin with only a
slight loss of potency. The regenerated material could be precipi-
tated again by heating under the same conditions as the first and
again reactivated by acid or alkali.

Blatherwick *et al.* (3) had earlier noted a similar precipitation
with their amorphous insulin and, although the conditions that they
had laid down for the precipitation have since been found to be of
much wider limits, there seems to be no doubt that they were
dealing with the same type of heat precipitation that we had
encountered later with crystalline insulin.

The most obvious change which accompanies heat precipitation
is the splitting out of ammonia from the insulin molecule as noted

by Freudenberg and coworkers (4) and by Jensen and Evans (5). This fact suggested to Jensen, after his isolation of glutamic acid from the hydrolysate of insulin, that the ammonia originated possibly in the amide group of glutamine. Jensen further suggested the interesting hypothesis that the formation of the heat precipitate might be due to some sort of ring closure between the amide grouping of glutamine and some adjacent amino or carboxyl group with the splitting out of ammonia. That the ammonia liberation might take place simultaneously with the formation of the heat precipitate without being directly connected with it is easily conceivable; for example, the liberation of ammonia during the heat coagulation of albumin has been shown by Sørensen (6) to be merely coincidental to the coagulation. It occurred to us that if the ammonia formation were intimately connected with the heat precipitation the rate of formation of ammonia might be expected to parallel that of heat precipitation, and, further, it seemed reasonable to expect that the formation of the precipitate should be conditioned by the liberation of a definite amount of ammonia and conversely that after a certain amount of ammonia had been split out the heat precipitate should make its appearance. We have therefore studied the liberation of ammonia from insulin in some detail.

This peculiar behavior of insulin to which we shall refer as heat precipitation appears at present to be the chief characteristic, aside from its physiological activity, which sets insulin apart from the host of animal and vegetable proteins. We have been unable so far to find any other substance not derived from insulin which will yield a heat precipitate under the conditions mentioned above for crystalline insulin. Furthermore, the inactivation of insulin by such mild reducing agents as cysteine and glutathione is accompanied by a loss of its ability to yield a heat precipitate (7). In view of all these suggestive facts, this heat precipitation reaction appeared to us to merit a much closer study.

<center>EXPERIMENTAL</center>

Heat Precipitation with Various Acids and Temperature Coefficient of Reaction—The observations that we had originally made on the heat precipitation of insulin with 0.1 N HCl were at 100°. In the present investigation we have extended these observations

to lower temperatures and to other acids.   The time required for the appearance of definite, flocculated particles with the formation of a thin gel at various temperatures with 0.1 N HCl was determined.   There is a considerable increase in the length of time required for the precipitate to form at lower temperatures; in fact, no precipitate was obtained at 50° even after heating for 4 days, only a few small flakes having separated out.   The temperature coefficient calculated from the data, however, showed considerable variation particularly at the higher temperatures.
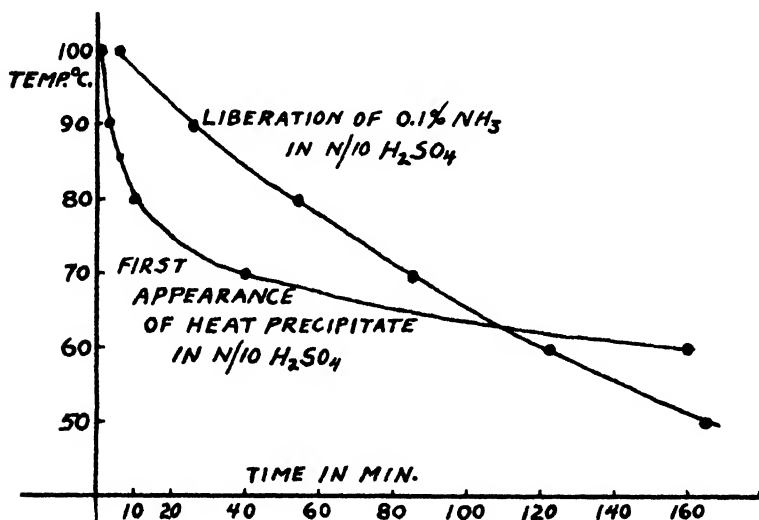
CHART I

0.1 N H_2SO_4, however, showed more promise as a precipitant for determining the temperature coefficient, because the heat precipitate formed in definite particles without gel formation.   The observation of the formation of the first trace of precipitate and the time required for its formation were easy to duplicate.   This superiority of H_2SO_4 is in accord with the finding of Gerlough and Bates (8) as is our observation that the speed of precipitation with H_2SO_4 is 3 to 5 times that with HCl.   From our data the temperature coefficient of the heat precipitation with H_2SO_4 was approximately 4 for a 10° rise in temperature which agrees with that calculated from the data of Gerlough and Bates.   The time required

for the first appearance of the heat precipitate at different temperatures is shown in Chart I.

In studying the action of more dilute acid we found that no heat precipitate was obtained with 0.01 N HCl even after heating for 8 hours at 100°. This was surprising in that at a slightly higher pH a precipitate resulted after only a few minutes heating. We found however that when a little salt was added, enough to make the solution about 0.17 M, the precipitation was complete in 5 to 10 minutes. This speeding up of the precipitation by a little salt was found to be true of the other precipitations as well.

The precipitation velocity with H_3PO_4 was found to be much slower than that with HCl or H_2SO_4. A heat precipitate was produced with 0.1 N H_3PO_4 at 100° in approximately 160 minutes and with 0.5 N in 60 minutes.

Acetic acid in concentrations from 0.01 N to 1.6 N failed to yield a heat precipitate with insulin when the solutions were heated at 100° for 8 hours.

The difference between HCl and H_2SO_4 as heat precipitants becomes more obvious in the more acid solutions. In solutions of acidity up to 1.5 N with respect to HCl the characteristic heat precipitate was formed, but with 2.0 N HCl no precipitate was given. With HCl from 0.5 N to 1.5 N insulin hydrochloride precipitated at room temperature, but as the temperature was increased insulin hydrochloride dissolved and the insulin then precipitated as the heat precipitate. That the failure to precipitate in the stronger acid was not a solubility phenomenon, but that the insulin had actually lost its ability to form the heat precipitate was shown by the fact that the insulin was not precipitated when the solution was subsequently made 1.0 N and again heated. The activity of the insulin was also lost.

In contrast to the behavior with HCl, heat precipitation is possible in H_2SO_4 with as high a concentration as 6 N. The so called insulin sulfate forms at room temperature but dissolves on heating and the insulin then comes out as heat precipitate.

Liberation of Ammonia in Relation to Heat Precipitation—The NH_3 liberated on heating the insulin with acid was determined by a method essentially the same as that described by Parnas and Heller (9) with certain modifications. In determining the amount of NH_3 split off from 10 mg. of insulin dissolved in 2 cc. of 0.1 N HCl

after heating for 1 hour at 100°, for example, the entire solution was transferred quantitatively with NH_3-free water to the distilling flask, neutralized with NaOH, and 2 cc. of paraffin oil were added, followed by 8 cc. of a borax buffer solution which contained 8.5 volumes of 0.05 M borax to 1.5 volumes of 0.1 N HCl. The NH_3 was then distilled over *in vacuo* at a pressure of 100 mm. of mercury into 1 N H_2SO_4 and determined colorimetrically by Nesslerization and comparison with $(NH_4)_2SO_4$ standards. With careful attention to the details of manipulation and by rigid exclusion of laboratory air from the apparatus during the distillation, by using heavy walled tubing for all connections, and by covering all joints with collodion, we were able to obtain almost theoretical recoveries of known amounts of NH_3. Repeated control determinations gave us definite proof that by our technique it was possible to estimate, with an error of less than 5 per cent, amounts of NH_3 ranging upwards from 0.005 mg., with much smaller error in amounts from 0.02 mg. to 0.05 mg. Control experiments on our insulin samples showed the absence of ammonium salts and further that NH_3 was not split from the insulin under the conditions of the distillation.

We intended at first to find the velocity constant of NH_3 removal but the impracticability of such a determination became apparent. We found repeatedly that there was liberated, although at a slower rate, considerable NH_3 even after the mother liquor became biuret-free. In other words, during the course of the precipitation, part of the NH_3 would be coming from the insulin in solution and part from the insulin already precipitated; hence, the constant would be meaningless. In the results that were obtained, however, poor correlation was shown between the NH_3 liberation and the heat precipitation, the rate of the former being much slower.

The rate of NH_3 liberation from insulin by 0.1 N H_2SO_4 was determined at 70°, 80°, 90°, and 100°. The concentration in all cases was 5 mg. per cc. For each point on the curves, either 10 mg. or 20 mg. samples were heated for the times and at the temperatures designated, and in every case the NH_3 determination was made on the entire sample. The data, checked by duplicate or triplicate samples, are shown on Chart II.

The disparity between the amount of NH_3 liberated and the amount of heat precipitate formed is illustrated in Chart I, a com-

parison of the time required for the cleavage of 0.1 per cent NH_3 at 10° temperature intervals with the time required for the first appearance of the heat precipitate at the same temperatures.   It is apparent that the temperature coefficients of the two reactions differ to the extent that at the higher temperatures precipitation precedes the liberation of 0.1 per cent NH_3, while below 60° the removal of 0.1 per cent NH_3 far precedes any trace of precipitation.

This lack of correlation is strikingly brought out by comparing the NH_3 liberated at 60° and at 90°.   At 60° for example it took
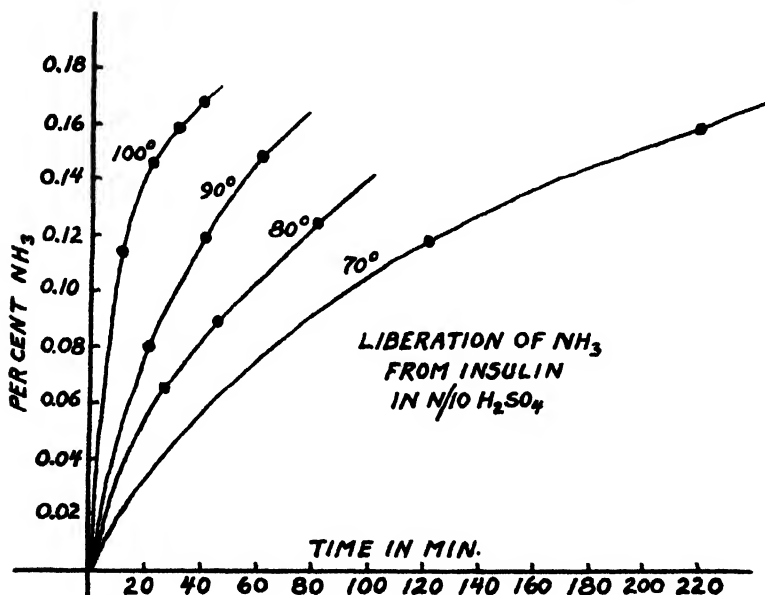
CHART II

160 minutes for the heat precipitate to begin to form in 0.1 N H_2SO_4 and at this point 0.11 per cent of NH_3 had been liberated, while at 90° the heat precipitate began to form within 3 minutes but the amount of NH_3 liberated was less than 0.03 per cent.   Although the rate of heat precipitation, as we have already shown, is much greater in H_2SO_4 than in HCl, the rate of NH_3 liberation by these two acids is the reverse. For example, at 100° 0.1 N HCl liberates 0.50 per cent NH_3 in 90 minutes whereas 0.1 N H_2SO_4 liberates 0.31 per cent under the same conditions. A more strik-

ing contrast is the fact that 0.01 N HCl liberates 0.45 per cent of NH_3 in 5 hours at 100° without a trace of heat precipitate while H_2SO_4 under the same conditions liberates only 0.35 per cent with an almost complete precipitation.

Inactivation and Loss of Heat Precipitability by Reducing Agents— As we have already reported (7), inactivation of insulin with cysteine or glutathione is attended by loss of heat precipitability. We have now extended these studies to reduction with H_2S and with NaCN.

The inactivation with H_2S was accomplished by allowing H_2S to bubble for 45 minutes through a $M/15$ Na_2HPO_4 solution of insulin containing 0.5 mg. of insulin per cc. Nitrogen was then bubbled through the mixture to free it from H_2S and the resulting solution was tested for potency. The material so inactivated was dissolved in 0.1 N HCl and placed in a boiling water bath. No heat precipitate was obtained.

After many preliminary experiments we found the minimum concentration of NaCN and the minimum length of time necessary to bring about inactivation of crystalline insulin. We wished to obtain the inactive product with as little change as possible in the molecule for it became quite evident that long continued action of the cyanide caused a more deep seated change resulting in an acid-insoluble product. This was also true of H_2S.

It was found that a solution containing 1 mg. of insulin and 2 mg. of NaCN became completely inactive within 3 hours. After 5 minutes of contact there was enough activity left so that when 0.1 mg. of insulin per kilo was injected into rabbits convulsions resulted. Within 30 minutes 0.1 mg. showed only a trace of activity but 0.5 mg. caused large blood sugar decreases. At 90 minutes the latter dosage produced only a slight fall and at 3 hours the insulin was completely inactivated. The insulin-cyanide solutions were injected directly, the amount of cyanide alone having been shown by control experiments to produce only a very slight rise if any in blood sugar. On heating the inactive material with acid no heat precipitate was obtained.

Nitroprusside tests were carried out as quantitatively as possible for comparative purposes on the above solutions at various time intervals. At 5 minutes the test was very weak and increased with length of time until a maximum was reached in a little over

an hour.   The increase in strength of the nitroprusside test was roughly proportional to the degree of inactivation.

Inactivation and Loss of Heat Precipitability by Acid and Alkali and Formation of Inactive Heat-Precipitable Material—As mentioned previously, heating insulin for a few minutes in 2 N HCl resulted in loss of both heat precipitability and activity.   Some experiments were therefore carried out at a lower temperature with 20 per cent HCl, in which the so called insulin hydrochloride is soluble, to see how readily activity and heat precipitability disappeared by this mild hydrolytic procedure.   A sample of insulin was allowed to stand in 20 per cent HCl at room temperature and samples were removed at 1 minute, 30 minutes, 5, 24, and 48 hours.   No activity and no heat precipitation were obtained with the material from the 24 and 48 hour samples.   Slight positive tests were obtained at the 5 hour point.

The behavior of insulin with dilute NaOH particularly brings out the extreme lability of the groupings necessary for heat precipitation as well as those necessary for activity.   Jensen and Evans (5) have already found that material treated with 0.03 N NaOH for 3 hours at 34° or 0.1 N NaOH at 50° for 1 hour does not yield a heat precipitate.   We have found that contact with 0.04 N NaOH for 12 hours at 25° would cause inactivation of the insulin as would be expected from previous reports (4, 5) and that this very mild treatment also caused disappearance of the heat precipitation reaction.

From the acid inactivations that have just been described, it is quite apparent that the groupings necessary for the formation of the heat precipitate can be modified readily by the action of acid so that the insulin loses its ability to yield a heat precipitate as well as its activity.   We have found though that, once the heat precipitate is formed, these groups involved in the formation of the heat precipitate are quite resistant to acid.   They are probably closed in a ring structure and, if the acid does not regenerate the material, the activity can be irreversibly destroyed before these groups are modified.   It was found that heating the heat precipitate with 6 N H_2SO_4 for 5 hours destroys all the activity but leaves an insoluble residue which can be regenerated by our standard procedure to an acid-soluble product.   This regenerated material although physiologically inactive can again be heat-precipitated.

Various attempts have been made to reactivate the material in order to see if it were potentially active. For example, the material after being dissolved at pH 11.4 was precipitated again by heating in 0.1 N HCl and again regenerated but no activity was demonstrable. Furthermore regeneration of solubility of the original modified heat precipitate by 20 per cent HCl led to no activity, and alkaline treatment according to Charles and Scott's (10) regeneration of acid-alcohol-inactivated insulin (11) was likewise negative.

This represents the first time that we have been able to isolate an inactive substance which would yield a heat precipitate under the conditions for heat precipitation of insulin in 0.1 N HCl. We are carrying out a more detailed study of this inactive heat-precipitable material, as a comparison of its composition and behavior with that of the original insulin may prove to be significant.

Regeneration of Heat Precipitate—The possibility of partial destruction of insulin in the regeneration of the heat precipitate by alkali led us to try to ascertain the lowest pH at which regeneration would occur. We also wished to establish definite standard conditions for regeneration that would be consistently duplicable. We therefore tried regeneration by various glycine-buffer mixtures. The lowest pH at which we could obtain practically complete solution of the heat precipitate was 11.4, and we therefore selected this pH in our standard conditions for regeneration. The procedure is as follows: 50 to 100 mg. of heat precipitate are suspended in 15 cc. of glycine-NaCl solution (12) at room temperature and 15.65 cc. of 0.1 N NaOH added. After allowing the mixture to remain at this pH for 5 minutes, it is then acidified. We have usually found a very slight trace of insoluble material.

Behavior of Regenerated Heat Precipitate—Having a standard set of conditions with which we could carry out regeneration of the heat precipitate we took up the study of the rate of precipitation of the regenerated material in order to see if the regenerated heat precipitate was different from the original insulin. We have found that the rate of precipitation of the regenerated material is far greater than that of the original, which fact indicates that we are dealing with a changed insulin. At temperatures from 70–100° the precipitation is almost immediate in 0.1 N HCl. Although the original insulin shows no precipitation at 50° even after heating

for days, the regenerated material gives a precipitate within a few minutes at 50°.   Still more remarkable, it gives a heat precipitate within a few hours at room temperature, and at 0° a heat precipitate overnight.   These precipitates behave like the original heat precipitate in solubility and regeneration.   It also became apparent in these studies that the case of precipitation of the regenerated material depends on the conditions under which the original precipitation has been carried out.   The stronger the acid and the greater the length of time required to bring about the original precipitation, the more readily is the second heat precipitate formed.   This can be brought out clearly by quoting the following experiments.   A sample of regenerated heat precipitate that had been formed by heating under the mild condition of 0.01 N acid and salt for 10 minutes would not yield a heat precipitate at room temperature although it would at 37° after being heated at this temperature overnight, whereas a sample of a regenerated heat precipitate that had been formed by heating the insulin in 0.1 N HCl for 2 hours gave not only a heat precipitate at room temperature, but also one at 0°.   That the alkaline regeneration was not the cause of the increased ease of heat precipitation of the regenerated material was demonstrated by many control experiments.

DISCUSSION

The difference between ordinary protein coagulation and the process of precipitation observed when insulin is heated in acid solution is quite obvious.   No protein of which we are aware can be coagulated by heat in acid of the concentrations that can bring about so readily the precipitation of insulin.   Denaturation of the common proteins without coagulation may be effected, of course, by either acid or alkali and, on subsequent adjustment of the pH to the acid concentration favoring agglutination, the protein coagulates very rapidly.   Insulin, on the other hand, does not show such behavior and if allowed to remain in contact with dilute alkali loses its ability to form a heat precipitate.

The coagulable proteins, owing to their high temperature coefficient of denaturation, exhibit an extremely large temperature coefficient of coagulation.   Coefficients from 58 to 9540 per 10° rise in temperature have been found (13).   Insulin, on the other hand, has a temperature coefficient of heat precipitation of only 4

per 10°, a coefficient well within the range observed for ordinary chemical reactions. It must be admitted however that there is no *a priori* reason why a coagulation should not have as low a temperature coefficient as 4.

Since there is as yet no definite evidence that we are dealing with a coagulation according to the generally accepted meaning of the term, we prefer to use the term heat precipitation. Freudenberg has used the term *Salzsäureinsulin*, but we feel that this terminology might lead to confusion with the term insulin hydrochloride as used by Dudley (14) to designate the insulin precipitate obtained at a concentration of 3 to 4 per cent HCl, a product entirely different from the precipitate formed in acid solution by heat.

We have attempted in these studies to throw some light on the possible relationship between the heat precipitation and the elimination of NH_3 which accompanies it. We have been unable to obtain any evidence favoring such a correlation. In fact our results are more in accord with the idea that the NH_3 liberation is merely coincidental to heat precipitation just as NH_3 liberation is incidental to protein coagulation. Sørensen (6) has shown that, for example, in the process of heat coagulation small amounts of NH_3 are liberated but concludes that the NH_3 so obtained has not arisen in the process of denaturation nor in the actual coagulation itself, but is rather the result of a very mild hydrolysis of the coagulated material.

In the previous work on the inactivation of insulin by cysteine and glutathione (7) it became apparent that the groupings involved in the heat precipitate could be modified readily with loss of this characteristic reaction. We have now been able to show that inactivation by H_2S or NaCN also results in loss of heat precipitability. The study of this relationship between inactivation and loss of heat precipitability has also been extended to inactivations by acid and alkali. Insulin inactivated in either of these two ways fails to give a heat precipitate. All of these studies indicate the lability of the molecule with respect to this heat precipitation reaction as well as to activity. The fact that these reactions seem to accompany each other led us to believe for a time that the groups involved in both behaviors were identical. The isolation however of an inactive heat-precipitable material from insulin decomposition has demonstrated that this cannot be the case. This repre-

sents the first time we have been able to isolate an inactive substance which would yield a heat precipitate under the conditions for heat precipitation in 0.1 N HCl or H_2SO_4. It might be pointed out, however, that so far we have found no active insulin preparation which would not yield a heat precipitate.

The fact that the active regenerated heat precipitate will yield again a heat precipitate more readily than the original insulin is definite evidence that the insulin has been modified by this precipitation. This finding indicates the way in which the data that we have obtained on the rate of precipitation of insulin can be utilized as a tool to detect changes in the molecule that might not otherwise be observed. The fact that this change has been brought about with retention of activity is an encouraging indication that we may be able to modify insulin still further without losing the hypoglycemic action. We are also hoping to study the action of enzymes on the active regenerated material since it may be possible that the behavior towards enzymes has also been modified.

SUMMARY

1. A study has been made of the heat precipitation of insulin with respect to the effect of various acids and acid concentration with special reference to the temperature coefficient of precipitation.

2. The amount of ammonia liberated from insulin under various conditions has been determined and compared with the amount of heat precipitate formed. No evidence has been obtained that ammonia removal is directly connected with the heat precipitation reaction; the results rather point to the fact that ammonia liberation is merely coincidental.

3. The effect of various agents on the heat precipitability and activity of insulin has been determined.

4. An inactive heat-precipitable material has been obtained by the action of acid on the heat precipitate itself.

5. Conditions for regenerating the activity and solubility of the heat precipitate yielding duplicable results have been worked out.

6. It has been demonstrated that the regenerated heat precipitate yields a heat precipitate again in 0.1 N HCl or H_2SO_4 more readily than does the original insulin.

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ON THE CEREBRONIC ACID FRACTION. II*

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The present experiments constitute a continuation of the fractionation of the acids obtained by the oxidation of cerebronic acid. The earlier paper[1] was concerned chiefly with the lower acids of the oxidation product, those acids being more readily separated from the mixture in crystalline form. We are now reporting similar experiments designed to isolate the acids of higher molecular weight. Many of the fractions dealt with here were obtained by fractionations that also gave rise to the lower members. They were held in reserve until a sufficient quantity for effective distillation had been accumulated.

From the fraction containing the higher fatty acids a fraction was obtained which had the molecular weight of tetracosanic acid with a melting point identical with that of lignoceric acid obtained from peanut oil, and with an identical mixed melting point. Evidence was also presented to show that the tetracosanic acid isolated from the oxidation product was not present in the cerebronic acid used for the oxidation experiments. Thus, the views expressed in our earlier article on the composition of cerebronic acid are now substantiated.

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* The present communication was ready for publication a year ago, prior to the appearance of the article by Klenk and Diebold (Klenk, E., and Diebold, W., *Z. physiol. Chem.*, **215**, 29 (1933)). It was withheld from publication pending the conclusion of investigations dealing with the same subject. The article is now presented in unchanged form without a discussion of the paper by Klenk and Diebold which will be given in a subsequent article.

[1] Taylor, F. A., and Levene, P. A., *J. Biol. Chem.*, **84**, 23 (1929).

The methods of determining the molecular weights, melting points, and solidification points were identical with those already described.[2,3]

The total oxidation product weighed 50 gm. At one stage of the separation most of the acids were grouped according to molecular weight, one of the combinations being made up of acids of which the average molecular weights were from 350 to 354. It weighed about 30 gm. and gave rise to the acids described in the earlier paper[1] as well as to acids of higher molecular weight.

The residues of Table III[1] together with the residues corresponding to the distillates of Table IV[1] were combined with a specimen of acid, the molecular weight of which was 354 (8.0 gm.), making in all 19.8 gm. The acid was esterified by boiling its solution in 99.5 per cent alcohol with sulfuric acid. The resulting ester was fractionally distilled. Fractions of highest and lowest volatility and a large middle distillate were removed and the process repeated on the middle distillate until all of the material had been distributed in fractions of the desired size. The molecular weights of the acids liberated from the distillates varied from 344 to 348. That of the final (most volatile) residue was 352. The weight of these fractions was 12.7 gm. The other residues of the distillation, which should have had considerably greater molecular weights, were collected together. They were united with similar residues making a total of 11.8 gm.

Since unaltered cerebronic ester would be concentrated in the residues, the material was first distilled as completely as possible. It was then fractionated in the manner described above. The acids liberated from the distillates, Distillates A, C, and D, weighed 7.0 gm. and had molecular weights of 353, 351, and 355. The first residue, Residue B (1.5 gm.), had a molecular weight of 366 and the second, Residue E (0.7 gm.), of 367. The distillates were crystalline, while the residues were in the form of dull powders.

The distillates (7.0 gm.) were united with other specimens of the same range of molecular weight and esterified for distillation. They were twice distilled as completely as possible and were then

[2] Taylor, F. A., and Levene, P. A., *J. Biol. Chem.*, **80**, 609 (1928).

[3] Taylor, F. A., *J. Biol. Chem.*, **91**, 541 (1931).

fractionated in the manner already described. All of the distillates were united (8.9 gm.) as were all of the residues (8.0 gm.). From the combined distillates, by repeating the fractionation, three distillates and two residues were obtained. The molecular weights of the distillates varied from 346 to 350 and the residues were identical at 357. They were all crystallized in the form of lustrous plates.

The combined residues (8.0 gm.) were united with Residue B, molecular weight 366 (1.5 gm.), and Residue E, molecular weight 367 (0.7 gm.), together with some other small fractions of similar material that had been accumulated. The material (12.7 gm.) was again esterified and twice distilled as completely as possible (residues 2.3 gm.). It was then fractionated in the usual manner. The acids from the distillates, Distillates A, C, and D (4.9 gm.), had molecular weights of 356 to 358. Residues B and E (3.6 gm.) both melted at 76.8–77.8° and solidified at 74.5°. They had molecular weights of 368 and 364 respectively. All but Residue B were nicely crystalline.

0.5010 gm. substance required 6 81 ml. 0 2 N NaOH
0.5007 " " " 6.88 " 0 2 " "

Residues B and E were then united with the crystalline acids obtained from reoxidized residues described in the previous paper[1] in Table II (Distillates B, C, and D). The acid (6.8 gm.) was repeatedly crystallized from ether (100 ml.) at 3–5°. Originally the mixture melted at 77.2–78.2° and solidified at 76.2–75.6°, while after eight crystallizations the melting point was 78.5–79.5° with solidification at 77–76.5°, the yield at the same time having been reduced to 3.8 gm. It was beautifully crystalline and had a molecular weight of 367.

0.4981 gm. substance required 6.78 ml. 0.2 N NaOH

The molecular weight being slightly low for tetracosanic acid, all of the material (6.8 gm.) was again collected, esterified, and fractionally distilled in the usual manner. The first distillate and the first residue were reserved. Distillates C and D (2.5 gm.) melted at 78–79° and solidified at 75.5–75°. Residue E (1.4 gm.) melted at 77–78° and solidified at 75.5–75°. The three fractions were united (3.9 gm.) and repeatedly crystallized from ether (75

ml.) at 4–5°.   After eight crystallizations the melting point was
79.5–80.5° with solidification at 77.5–77°.   The acid crystallized
in lustrous plates.   The yield was 1.7 gm.   Four further crystalli-
zations brought about no change in the melting point.

0 1000 gm. substance: 0.2863 gm. CO_2 and 0.1175 gm. H_2O
0 5013 "   "   : 6 77 ml. 0.2 N NaOH
        $C_{24}H_{48}O_2$.   Calculated.   C 78 26, H 13.04, mol. wt. 368
                Found.   " 78.07, " 13.15, "   " 370

The acid was finally mixed in approximately equal proportion
with lignoceric acid from peanut oil and the melting point of the
mixture determined.   The result is shown in Table I.

TABLE I

*Melting Points of Tetracosanic Acid from Cerebronic Acid, of Lignoceric Acid
from Peanut Oil, and of Their Mixture*

Substance	Melting point	Solidification
	°C.	°C.
Tetracosanic acid from cerebronic acid...	79 5–80.5	77.5–77
Lignoceric acid from peanut oil	79.5–80 5	77.5–77
1:1 mixture (approximate)	79 5–80 5	77 5–77

Lignoceric acid is a constituent of the cerebroside cerasin.   The
cerebronic acid used in this investigation was prepared from a
sample of partially purified phrenosin which contained some cera-
sin.   The significance of the isolation of lignoceric acid from the
oxidation product is therefore dependent on an examination of the
cerebronic acid for the presence of lignoceric acid before oxidation.
The following fractionations indicate that lignoceric acid cannot
be present in the cerebronic acid in appreciable concentration.

A sample (10 gm.) of the cerebronic acid was esterified in the
usual manner.   It melted at 64–65° and rotated in pyridine as
follows:

$$[\alpha]_D^{26} = \frac{+0.30° \times 100}{2 \times 6.02} = +2.5°$$

The ethyl ester was distilled into three fractions, two distillates
and a residue.   The first distillate melted at 65–66° and had a
rotation of +2.3° in pyridine:

$$[\alpha]_D^{24} = \frac{+0.28° \times 100}{2 \times 6.08} = +2.3°$$

The second fraction of the distillate melted at 64.5–65.5°. Its rotation in pyridine was +2.5°.

$$[\alpha]_D^{24} = \frac{+0.30° \times 100}{2 \times 6.02} = +2.5°$$

The distillation residue melted at 59–60°. The solution in pyridine was not suitable for rotation.

The experiment is complicated by the condensation of cerebronic acid to material of higher rotation on exposure to heating.

TABLE II

Physical Properties of Fractions of Cerebronic Acid

Fraction	Yield	Melting point	Solidification	Rotation (pyridine)		
				Temperature	α	Concentration	$[\alpha]_D^{24}$
	gm.	°C	°C.	°C.	*degrees*	*gm. per 100 ml.*	*degrees*
A	0 8	100 5 101 5	99 5–99	24	+0 16	3 32	+2.4
B	1 0	100 5–101.5	99 5–99	24	+0 16	3.36	+2 4
C	2.9	100 5–101 5	99 5–99	24	+0 16	3 32	+2.4

However, the melting point of the first distillate is not depressed as would be expected by the concentration of any lignoceric acid in the most volatile fraction.

A further attempt to separate lignoceric acid consisted of the fractional saponification of the ester as described by Thierfelder.[4] The cerebronic acid was first thoroughly saponified in solution in alcohol-toluene. It was then esterified in the usual manner. The ethyl ester (5 gm.) in 400 ml. of ether at room temperature was treated with 1 equivalent of 0.5 N alcoholic sodium hydroxide and allowed to stand for 25 minutes when, after settling, the supernatant solution remained clear. The sodium salt (Fraction A) was then filtered and washed with ether. Another equivalent of alkali was added to the filtrate and the mixture again allowed to stand until precipitation was complete. The soap (Fraction B) was separated and the solution (Fraction C) was evaporated.

[4] Thierfelder, H., *Z. physiol. Chem.*, **85**, 35 (1913).

The three fractions were saponified and the acids liberated from the salts with concentrated hydrochloric acid in the presence of ether. They were finally crystallized from acetone at $-1°$. The result of the examination of the acid is given in Table II.

The cerebronic acid from which these fractions were derived is described in the earlier paper.[1] It melted at 99.5–100.5° and solidified at 99–98°. Its rotation in pyridine was $+3.7°$.

Lignoceric acid should have been concentrated in Fraction C. Since there is depression neither of the melting point nor of the rotation, its absence may be assumed.

CEREBRONIC ACID. IX

By P. A. LEVENE and P. S. YANG

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The nature of cerebronic acid has been a matter of controversy between Klenk[1] and our laboratory[2] for so long a time that a reinvestigation of the problem seemed desirable. Hence, Klenk and ourselves simultaneously undertook the task, employing a much larger quantity of starting material than on previous occasions. The essential points of disagreement, as they stood before Klenk's last publication, were the following.

This author claimed that the acid isolated from phrenosin by Thudichum,[3] having the composition $C = 78.5$ and $H = 13$ (to which Thierfelder[4] later assigned the structure $C_{25}H_{50}O_3$), on oxidation yielded the acid $C_{23}H_{46}O_2$. Inasmuch as there was evidence for the assumption that cerebronic acid had the structure of an α-hydroxy acid, Klenk assigned to cerebronic acid the structure of α-hydroxytetracosanic acid, $C_{24}H_{48}O_3$. Klenk, however, failed to prepare a sample of the acid giving a titration value corresponding to $C_{24}H_{48}O_3$; on the contrary, the value agreed better with a molecular weight corresponding to $C_{25}H_{50}O_3$. This discrepancy Klenk explained by the assumption of a lactide formation.

On the other hand, from experiments of Taylor and Levene[5] and of Levene and Heymann,[6] it seemed that the oxidation product

[1] Klenk, E., *Z. physiol. Chem.*, **174**, 214 (1928).

[2] Levene, P. A., and Jacobs, W. A., *J. Biol. Chem.*, **12**, 381 (1912). Levene P. A., and West, C. J., *J. Biol. Chem.*, **18**, 477 (1914); **26**, 115 (1916). Taylor, F. A., and Levene, P. A., *J. Biol. Chem.*, **80**, 609 (1928).

[3] Thudichum, J. L. W., Die chemische Konstitution des Gehirns, Tübingen, **194**, 195 (1901).

[4] Thierfelder, H., *Z. physiol. Chem.*, **43**, 21 (1904).

[5] Taylor, F. A., and Levene, P. A., *J. Biol. Chem.*, **102**, 535 (1933).

[6] Levene, P. A., and Heymann, K., *J. Biol. Chem.*, **102**, 1 (1933).

consisted of a mixture of acids ranging from C_{22} to C_{24}.   In the latest publication of Klenk and Diebold, the oxidation product was found to consist of a mixture of the acid $C_{23}H_{46}O_2$ with acids having a molecular weight *lower* than that of C_{23} but they did not report the presence of acids with higher molecular weight.   The separation of the acids was not continued beyond a single fractional distillation of their esters.   The origin of these lower fatty acids Klenk attributed to further oxidation of one and the same cerebronic acid with the composition of $C_{24}H_{48}O_3$.

Our present investigation was begun a year ago.   The points which it aimed to elucidate were the following: (1) Is it possible to obtain among the oxidation products of cerebronic acid an acid having a molecular weight corresponding to $C_{24}H_{48}O_2$ when the starting material does not contain any detectable admixture of that acid?   (2) Can the molecular weight 396 to 400, generally found for cerebronic acid, be attributed to lactide formation?   (3) Do the higher α-hydroxy acids on oxidation with permanganate yield more than one acid of the type $C_nH_{2n}O_2$?

Purification of Cerebronic Acid—It was realized that the cerebronic acid used in the previous work in our laboratory might have been contaminated with the so called lignoceric acid and that the acid $C_{24}H_{48}O_2$ found previously might have been attributed to this cause.   True, the proportion of this acid found in the oxidation product was too large to have escaped detection, yet this possibility had to be investigated with greater rigor than had been attempted hitherto.   For the purpose of purification, a procedure was followed which was based on the velocity of hydrolysis of the esters of the higher α-hydroxy fatty acids, a method introduced by Thierfelder.   Our crude cerebronic acid was esterified and to the ethereal solution of the ester a small excess of an alcoholic solution of sodium hydroxide was added.   After standing at room temperature during 15 minutes, the soaps were filtered off.   To the filtrate more alcoholic sodium hydroxide was added and saponification continued by refluxing on the steam bath for 8 hours.   The soaps of each fraction were exhaustively extracted with acetone, and the free acids liberated and analyzed.   The hydroxy acid fraction was reesterified and resaponified in the same manner as in the first operation and the procedure was repeated until practically the entire quantity of the esters was saponifiable in 15

minutes, leaving in the mother liquor only an inappreciable residue, which on saponification and subsequent liberation of the acid gave a substance of the molecular weight approaching that of $C_{25}H_{50}O_3$. About fifteen operations were required before this degree of purity was reached. The physical constants of the individual samples are given in Table I.

Molecular Weight of Cerebronic Acid—Two methods were employed for the purpose of establishing the molecular weight of the substance.

First Method—It was stated above that the high molecular weight of cerebronic acid was attributed by Klenk to contamination with its lactide. To test this possibility, a sample of the cerebronic acid was dissolved in a solution containing 1.5 gm. of sodium hydroxide in equal parts of toluene and methyl alcohol and the solution was refluxed on the steam bath for 48 hours. In this solvent the soaps remain in solution at the temperature of the experiment and thus conditions are maintained permitting complete hydrolysis of the lactide portion of the material. The titration value of the acid obtained after this treatment was practically unaltered, corresponding again to a molecular weight of 398; the acid compared with the starting material had, however, a lower optical rotation ($+1.98°$) and slightly lower melting point (97–99°) and solidification point (94-95°).

Second Method—By the second method it was aimed to prepare a derivative of cerebronic acid incapable of lactide formation. The methoxy derivative seemed the most promising. In addition, it was hoped that the comparatively low boiling point of the methyl methoxycerebronic ester would permit of its fractionation. Indeed, four fractions which differed only slightly in their melting points and composition but which differed somewhat more in their specific rotations (as may be seen from Table IX) were obtained. Likewise, the acids derived from each fraction differed little from each other in their melting points, but somewhat more in their solidification points, the lowest boiling fraction having a solidification point of 64-65° and the highest 67-68°. More significant, however, was the fact that in the higher fraction the solidification point came nearer to the melting point than in the lower fraction, as may be seen from Table X. In a general way, the numerical difference between the melting and solidification points of all

fractions may probably serve as an indication of the heterogeneity of the substances. The titration value of every fraction corresponded to a molecular weight of 412 which would suggest that the parent substance was an acid having a molecular weight of 398; hence, the value 398 generally found on titration of cerebronic acid cannot be attributed to lactide formation and argues against the assumption of 384 as the molecular weight of cerebronic acid.

Oxidation of Cerebronic Acid—140.0 gm. of the acids were oxidized and 116.0 gm. (or 83 per cent) of crude oxidation product were obtained. In the analysis of the oxidation products of the acid, special care was taken to remove the unchanged acid as completely as possible. Two procedures were employed for this purpose.

One was based on the same principle as that employed in the purification of cerebronic acid, namely on the high velocity of the hydrolysis of the hydroxy acids by means of an alcoholic solution of sodium hydroxide, the difference being that attention was concentrated on the fraction hydrolyzable at a slower rate and higher temperature. This fraction was resaponified and reesterified repeatedly until, on one hand, the hydroxy acid fraction was reduced to an insignificant minimal quantity and, on the other hand, the simple fatty acid acquired the character of bright lustrous platelets.

The second method consisted in reoxidation of the product of the first oxidation and again obtaining a fraction which crystallized in bright glistening platelets.

The acids were then esterified and the esters fractionated in five fractions, the fractions of similar resolidification points being grouped together and each group again fractionated. The operation was repeated seven times.   Ten fractions were obtained. 1 gm. of each fraction was converted into the free acids and the molecular weights and melting points of these acids are given in Table V.   From Table V it may be seen that the molecular weights of the acids of different fractions varied between 340 and 380, the latter fraction being very small.

The acids having a molecular weight of 340 to 343 (corresponding to $C_{22}H_{44}O_2$) amounted to about 7 per cent.   Those having a molecular weight of 352 to 357 amounted to about 32 per cent and were recrystallized several times from ether.   The final product

had a molecular weight of 354, a melting point of 77–78°, and a solidification point of 76–75°. An intermediate fraction of the molecular weight of 358 to 361 amounted to about 18 per cent. The acids of molecular weight 367 to 371 (corresponding to $C_{24}H_{48}O_2$) amounted to about 32 per cent and 8 gm. of these acids were recrystallized five times from ether, final crystallization being carried out at a temperature of 28°. The melting point was 80.5–82°, the solidification point 79.5–78.5°.

The yield of the higher fatty acid, $C_{24}H_{48}O_2$, was too significant to have been present in the cerebronic acid without noticeably lowering its molecular weight. Thus, our results seem to be in harmony with the results of Taylor and Levene[5] and of Levene and Heymann.[6] All these investigations lead to the conclusion that on oxidation of cerebronic acid the fatty acids C_{22}, C_{23}, and C_{24} are obtained and hence, that cerebronic acid is *not* a homogeneous substance.

Klenk's conclusions are in disagreement with those reached in this laboratory. What are the experimental facts reported by Klenk and Diebold[7] in their recent publication? 33.5 per cent of the total acids obtained by them on oxidation of cerebronic acid had a molecular weight below 354. The rest had a molecular weight of about 357. One fractional distillation only was carried out. On fractional crystallization of the higher fraction an acid of molecular weight and of the melting point of *n*-tricosanic acid was obtained. In one particular, Klenk's observations definitely agree with ours; namely, with respect to the presence of the lower fatty acids. Had the fractionation of the higher fraction been carried out with greater rigor, the fraction corresponding to the tetracosanic acid might likewise have been discovered. However, Klenk attributes the origin of the lower fatty acids to a higher degree of oxidation of cerebronic acid. In the lower hydroxy fatty acids the formation of more than one oxidation product was observed by Witzemann.[8] On the other hand, Taylor and Levene[2] on oxidation of α-hydroxylignoceric acid could not detect an acid lower than tricosanic. It will be shown in the following paper that α-hydroxystearic acid on oxidation with permanganate yielded no fatty acid lower than margaric acid. Thus, it seems

[7] Klenk, E., and Diebold, W., *Z. physiol. Chem.*, **215**, 79 (1933).

[8] Witzemann, E. J., *J. Biol. Chem.*, **95**, 219 (1932).

to us that Klenk's latest experimental results approach nearer our own and like ours, should lead to the conclusion that there exists *more* than one hydroxy acid in the "cerebronic" acid. Whether or not hydroxy acids with hydroxyl group in a position other than α (such as β or γ) are present in cerebronic acid remains to be established.

<div align="center">EXPERIMENTAL</div>

Purification of Cerebronic Acid—Four different lots of crude cerebronic acid, obtained from the hydrolysis of mixed cerebrosides, were purified rigorously and separately by esterification and saponification in the cold. This operation was repeated until the quantity of soap remaining in the filtrate from cold saponification was negligible and the acid obtained from it had a composition approaching that of cerebronic acid. Complete removal of all the lignoceric acid present required from ten to fifteen operations of esterification and saponification for each lot of the crude acid. The details of a typical purification are as follows:

A solution of 45 gm. of cerebronic acid in 1000 cc. of absolute methyl alcohol containing 25 cc. of concentrated sulfuric acid was refluxed on a steam bath for 5 hours. On cooling to about $+5°$ the ester crystallized in needles and was filtered by suction. The filtrate was concentrated under reduced pressure and then shaken with a little ether. The ether extract combined with the solution of the ester in about 800 cc. of ether was shaken with water until free from sulfuric acid. To the ethereal solution was then added with stirring a cold solution of 8 gm. of sodium hydroxide in about 300 cc. of methyl alcohol. The saponification took place in about 5 minutes and was complete in about 10 minutes. The sodium salt resulting from the saponification was in the form of a thick paste which was allowed to stand at room temperature for 15 minutes, after which it was filtered by suction. The solid was thoroughly washed successively with alcohol, ether, and acetone, and then suspended in ether in a separatory funnel, and decomposed by means of concentrated hydrochloric acid. The ether extract was freed from hydrochloric acid by washing with water, dried over sodium sulfate, and the ether evaporated off. The remaining acid was dissolved in acetone and on cooling gave an amorphous powder. The properties of the four lots of the purified acid are tabulated in Table I

TABLE I

A. Cerebronic Acid ($C_{25}H_{50}O_3$), Molecular Weight 398.4

Lot No.	Amount	Melting point	Solidification point	$[\alpha]_D^t$	Molecular weight
	gm.	*°C.*	*°C.*	*degrees*	
1	30	100–102	99–98	+3 95	397
					399
2	85	99–101	96–97	+3 69	398
					398
3	31	98–99	95 96	+3 65	400
					401
4	100	100–101	97–98	+3 34	400
					400

B. Data for Specific Rotation and Molecular Weight Determinations

Lot No.	Rotation in pyridine and 2 dm. tube			Molecular weight determination	
	Substance in 5 cc. pyridine	α_D, 2 dm tube	t	Substance	0 1 N NaOH
	gm.	*degrees*	*°C*	*gm.*	*cc.*
1	0 1074	+0 17	24	0 1547	3 89
				0 1502	3 76
2	0 1153	+0 17	26	0 1615	4 06
				0 1461	3 67
3	0 0685	+0 10	26	0 0823	2 06
				0 1446	3 60
4	0 1349	+0 18	28	0 1604	4 01
				0 1650	4 11

C. Analytical Data

Lot No.	Substance	CO_2	H_2O	C	H
	mg	*mg*	*mg*	*per cent*	*per cent*
1	4 100	11 325	4 605	75 32	12 56
2	3 800	10 535	4 325	75 65	12 73
3	4 765	13 185	6 240	75 45	12 30
4	3 905	10 740	4 455	75 00	12 70
Calculated for $C_{25}H_{50}O_3$.				75 33	12 50

Hot Saponification of d-Cerebronic Acid—In order to make certain that the high molecular weight was not caused by lactide formation, 4 gm. of Lot 3 (Table I, *A*) were dissolved in a mixture of 150 cc. of toluene and 150 cc. of methyl alcohol containing 1.5 gm.

of sodium hydroxide, and refluxed on a steam bath for 48 hours, the sodium salt remaining in solution. On cooling, the sodium salt crystallized out and was filtered off, boiled with acetone, and then decomposed with concentrated hydrochloric acid. The yield of free acid was 3.60 gm. Melting point, 97–99°; solidification point, 94–95°.

0.2000 gm. required 5.03 cc. 0.1 N NaOH
Mol. wt. found, 398

$$[\alpha]_D^{28} = \frac{+0.10° \times 5}{2 \times 0.1286} = +1.98° \text{ (in pyridine)}$$

Oxidation of Cerebronic Acid—The oxidation of cerebronic acid was carried out according to Levene and Taylor,[9] except that powdered potassium permanganate was used instead of the solution of potassium permanganate in acetone. It was also found that concentrated hydrochloric acid alone (without the aid of sodium bisulfite) decomposed the brown mixture of the potassium salt and manganese dioxide formed. 140 gm. of cerebronic acid were oxidized in six lots. The amount of potassium permanganate was calculated on the basis of a 25 per cent excess over the requirement for the oxidation of 2 carbon atoms (16.4 gm. of the reagent for 10.0 gm. of the acid). The properties of the crude oxidation product are tabulated in Table II.

Purification of Oxidation Product of Cerebronic Acid

Procedure A.   By Repeated Esterification and Saponification in the Cold—Lots 1 to 3 (Table II, *A*) were subjected to the procedure of esterification and saponification in the cold as described for the purification of cerebronic acid. Lots 1 and 3 were purified together, and Lot 2 separately. After the fourth operation the acid obtained was crystalline.

The yield from Lots 1 and 3 combined was 20 gm. It melted at 75–76° and resolidified at 72–73°. Its molecular weight was 362 (0.1958 gm. of substance required 5.41 cc. of 0.1 N NaOH).

The yield of Lot 2 was 20 gm. Its melting point was 75–76° and resolidification point 72–73°. Its molecular weight was found

[9] Levene, P. A., and Taylor, F. A., *J. Biol. Chem.*, **84**, 23 (1929).

to be 364 (0.1970 gm. of substance required 5.40 cc. of 0.1 N NaOH).

Procedure B.   By Repeated Oxidation—Lots 4 to 6 (Table II, *A*) were separately subjected to reoxidation in order to insure com-

TABLE II

A. Crude Oxidation Product of d-Cerebronic Acid

Lot No.	Cerebronic acid	Yield	Melting point	Solidification point	Molecular weight
	gm.	*gm.*	*°C.*	*°C.*	
1	20	16.0	73–75	70–71	369
					370
2	40	33.5	74–76	70-71	383
					382
3	20	17.0	74–76	70–71	367
					367
4	20	14 5	74–76	70·71	370
					371
5	20	18 0	74–76	70–71	384
					380
6	20	18 0	74–76	70–71	365
					366

B. Data for Molecular Weight Determinations

Lot No	1	2	3	4	5	6
Substance, *gm*.....	0 1840	0.1537	0 1182	0.1379	0 1620	0 1530
	0 1490	0 1413	0.1274	0.1488	0 1730	0 1481
0.1 N NaOH, *cc*....	4.99	4 01	3 22	3 72	4 22	4 19
	4.03	3 70	3 47	4.01	4 48	4 04

plete oxidation.   The yield of the crystalline acid from Lot 4 was 16.5 gm.   It melted at 73-74° and resolidified at 70-71°.

Sample *1*—0.1392 gm. required 3.91 cc. 0.1 N NaOH
    "   *2*—0.1381 "   "   3.86 " 0.1 "   "
Mol. wt. found: Sample 1, 356; Sample 2, 358

The yield of crystalline acid from Lot 5 was 14.5 gm.   Its melting point was 74-76°, and resolidification point 71°.

Sample *1*—0.1104 gm. required 3.06 cc. 0.1 N NaOH
    "   *2*—0.1588 "   "   4.39 " 0.1 "   "
Mol. wt. found: Sample 1, 360; Sample 2, 360

The yield of crystalline acid from Lot 6 was 15.5 gm. It melted at 73–75°, and resolidified at 70–71°.

0.1148 gm. substance required 3.19 cc. 0.1 N NaOH
Mol. wt. found, 360

Procedure C. By Repeated Fractional Distillation of the Ethyl Ester of the Oxidation Product. Lot A—The oxidation products purified by Procedure A were combined and esterified by refluxing for 5 hours with 1 liter of absolute alcohol containing 50 cc. of

TABLE III

First Fractionation of Ethyl Ester of Oxidation Product of d-Cerebronic Acid

Fraction No.	Tempera-ture	Bath tem-perature	Pressure	Amount	Melting point	Solidification point
	°C.	°C.	mm	gm	°C	°C
Lot A						
1	190–204	250–260	0 13–0 2	6 3	51 5–52 5	49 0–50 0
2	190–204	250–260	0 13–0 2	6 8	51 5–52 5	49 0–50 0
3	190–204	250–260	0 13–0 2	6 5	52 0–53 0	49 0–50.0
4	190–204	250–260	0 13–0.2	8 0	52 0–53 0	49 5–50 5
5	190–204	250–260	0 13–0.2	7 8	53 0–54 0	51 0–52 0
Residue				2 0	53 5–54 5	52 5–53 0
Lot B						
1	185–195	250–260	0 1 –0 2	8 1	49 0–50 0	47 0 48 0
2	185–195	250–260	0 1 –0.2	7 7	50 0–51 0	48 0–48 5
3	185–195	250–260	0 1 –0 2	5 4	50 5–51 5	48 5–49 5
4	185–195	250–260	0 1 –0.2	6 4	52 0–53 0	49 0–50.0
5	185–204	250–260	0 1 –0 2	6 8	52 5–53 5	50 5–51 5
Residue				2.0	53 5–54.5	51 5–52.0

concentrated sulfuric acid. The ethyl ester thus obtained and recrystallized once from absolute alcohol melted at 54–56°, and resolidified at 50–51°. It was then fractionated as shown in Table III.

Lot B—The oxidation products purified by Procedure B were combined and esterified separately. The substance melted at 53–55° and resolidified at 50–51° and was fractionated as shown in Table III.

The different fractions of Lots A and B (Table III) of the ester were grouped according to their resolidification points and

again fractionated. After regrouping and then refractionating for the seventh time, the following ten fractions, the properties of which are recorded in Table IV, were obtained.

1 gm. portions of each of the ten fractions of ester (Table IV) were saponified with 50 cc. of methyl alcohol containing 3 cc. of 5 N sodium hydroxide, and the respective sodium salts were converted to the free acid, the properties of each being recorded in Table V, and their analyses in Table VI.

TABLE IV

Seventh Fractionation of Ethyl Ester of Oxidation Product of d-Cerebronic Acid

Fraction No	Temperature	Bath temperature	Pressure	Amount	Melting point	Solidification point
	°C.	°C.	mm	gm.	°C.	°C.
1	170–180	205–215	0 15	5 0	47–48	45 8
2	160–180	205–220	0 08	5 4	48–48 5	47 2
3	165–185	215–230	0 05	4.3	51	49 5
4	170–185	220–235	0 15	5 5	51	49 5
5	178–190	210–220	0 20	7 7	51	49 5
6	183–194	210–220	0 20	13 8	51	49.8
7	186–195	210–230	0 20	7 0	53	51 5
8	184–195	210–220	0 20	7 8	53	51 5
9	186–207	220–230	0 20	7 6	54	52.5
10	194–200	225–240	0 20	7 0	54 5	53 0

Purification of Acids—9.0 gm. of Fractions 2 to 5 (Table V) were combined and recrystallized five times from ether at a temperature of 10°. The melting point showed practically no change. The final product had a melting point of 77–78° and solidification point of 76–75°.

4.090 mg. substance: 11.638 mg. CO_2 and 4.700 mg. H_2O
0.2034 gm. " required 5.854 cc. 0.1 N NaOH (the end titration being carried out with 0.05 N NaOH)

$C_{23}H_{46}O_2$. Calculated.   C 77.88, H 13.08, mol. wt. 354
      Found.   " 77.60, " 12.85, "   " 356

8 gm. of the combined Fractions 7 to 9 (Table V) were recrystallized from ether, three times at 10° and twice at 28°. The melting point of the substances from the last two crystallizations showed

TABLE V

Free Acid from Oxidation Product of d-Cerebronic Acid

Fraction No.	Yield of acid from 1 gm. ester	Melting point	Solidifica-tion point	Molecular weight determination		
				Substance	0 1 N NaOH	Molecular weight
	gm.	*°C.*	*°C.*	*gm.*	*cc.*	
1	0 70	71.5	70 0	0 1044	3 06	340
				0 1166	3.40	343
2	0 72	73 0	71.0	0 1026	2 91	352
				0 0914	2.59	353
3	0.80	74.5	73 0	0 0892	2 51	355
				0 0840	2 35	357
4	0 79	74 5	73 0	0 1318	3 70	356
				0 1286	3.62	357
5	0 82	74 5	73 0	0 1364	3 82	357
				0 1480	4 15	356
6	0 80	76 0	74 0	0 1491	4 13	361
				0 1271	3 55	358
7	0.80	77 5	75 5	0 1266	3 45	367
				0 1228	3 33	368
8	0 84	77 5	75 5	0 1539	4 19	367
				0 1353	3 69	367
9	0 70	77 5	75 5	0 1426	3 84	371
				0 1619	4 38	370
10	0 82	78 0	76 0	0 1289	3 38	381
				0 1310	3 44	381

Calculated for $C_{22}H_{44}O_2$ 340 4

" " $C_{23}H_{46}O_2$ 354 4

" " $C_{24}H_{48}O_2$ 368 4

TABLE VI

Analytical Data for Oxidation Products of d-Cerebronic Acid (Table V)

Fraction No.	Found					Calculated percentage composition
	Sub-stance	CO_2	H_2O	C	H	
	mg.	*mg.*	*mg.*	*per cent*	*per cent*	
1	3 380	9.600	3 980	77 45	13.17	$C_{22}H_{44}O_2$ (340 4)
2	3.755	10 705	4 355	77 81	13 00	C = 77.65
3	3 900	11 130	4.540	77.82	13 03	H = 12 94
4	3 825	10 950	4.490	78 06	13 13	$C_{23}H_{46}O_2$ (354 4)
5	3.778	10.809	4.430	78 02	13 12	C = 77 88
6	4.065	11 615	4.750	77 91	13 07	H = 13 08
7	3 960	11.245	4.630	77 44	13 08	$C_{24}H_{48}O_2$ (368 4)
8	3 260	9.350	3 840	78 21	13 18	C = 78 18
9	3.770	10 800	4 380	78 12	13 00	H = 13.13
10	3.668	10 515	4.295	78.17	13.10	

no change. The final product had a melting point of 80.5–82°
and a solidification point of 79.5–78.5°.

4.108 mg. substance: 11.795 mg. CO_2 and 4.855 mg. H_2O
0.2024 gm.   "   required 5.46 cc. 0.1 N NaOH (the end titration
being carried out with 0.05 N NaOH)
$C_{24}H_{46}O_2$. Calculated. C 78.26, H 13.04, mol. wt. 368
      Found.   " 78.29, " 13.22, " " 370

Preparation of Methyl Methoxycerebronate—30 gm. of methyl
cerebronate were suspended in about 50 cc. of acetone with 65 gm.
of methyl iodide, and then 50 gm. of silver oxide were added in
small portions at intervals of 30 minutes. The reaction mixture
was heated in a bath at about 50°, and stirred by a mechanical

TABLE VII

Methyl Methoxycerebronate

Fraction No	Tempera- ture	Bath tem- perature	Pressure	Amount	Melting point	Solidifica- tion point
	°C	°C.	mm.	gm.	°C.	°C.
1	190–208	230–250	0 20	5 7	56–57	50–54
2	200–215	240–250	0 20	12 0	58–59	50–54
Residue..	290			10 5	59	57

stirrer, the treatment occupying 5 hours. The product was then
thoroughly extracted with acetone. After removing acetone by
distillation, the methyl methoxycerebronate remained in the flask
as an oil which solidified on cooling.

The methylation was repeated by dissolving in methyl iodide
and treating with the same amount of silver oxide under the condi-
tions described above. The methylated product was then frac-
tionated as shown in Table VII.

Fractions 1 and 2 (Table VII) were combined and refractionated.
The ester was fractionated a third and fourth time. The data
obtained for the fourth fractionation are recorded in Tables VIII
and IX.

The residue from the first distillation was then dissolved in a
50 per cent mixture of methyl alcohol and ether and shaken with
norit. After filtering and then removing the ether, the substance

came out as a white amorphous powder.   A yield of 8 gm. was obtained.   The substance melted at 59° and resolidified at 56°, but the melting and resolidification points were not sharp.

TABLE VIII
Fourth Fractionation of Methyl Methoxycerebronate

Fraction No.	Temperature	Bath temperature	Pressure	Amount	Melting point	Solidification point
	°C.	°C.	mm.	gm.	°C.	°C.
1	187–197	220–230	0.15	2 5	58 0	51–52
2	180–190	230–240	0 15	6.6	58.0	51–54
3	190–210	230–250	0 15	3 1	58 5	51–53
4	210–215	230–245	0.15	4 2	59.0	51–54

TABLE IX
Specific Rotation of Methyl Methoxycerebronate

Fraction No.	Substance in 5 cc. pyridine	α_D	t	Tube	$[\alpha]_D^t$
	gm.	degrees	°C.	dm	degrees
1	0 0655	+0.47	26	2	+17 94
2	0 0970	+0.70	26	2	+18 04
3	0 0621	+0 49	26	2	+19 70
4	0.0742	+0 57	26	2	+19 20

Analytical Data

Fraction No.	Substance	CO_2	H_2O	C	H	Substance	AgI	OMe
	mg.	mg.	mg.	per cent	per cent	mg.	mg.	per cent
1	3.790	10 525	4 280	75 74	12 64	9 281	9 890	14.06
2	3.895	10.820	4.400	75.75	12 63	10 100	11 115	14 52
3	3.980	11 002	4 530	75.70	12 73	8 231	8 886	14.25
4	4.043	11 320	4.600	76 35	12 41	9 040	10 084	14 72
$C_{27}H_{54}O_3$ (425.4) calculated....				75 98	12.76			14.54

1 gm. of the crystallized substance was saponified with 50 cc. of methyl alcohol containing 4 cc. of 5 N sodium hydroxide.   The free acid obtained melted at 89–93° and resolidified at 84–90°.

Sample A—0.1562 gm. required 3.83 cc. 0.1 N NaOH
"   *B*—0.1748 "   "   4.28 " 0.1 "   "
Mol. wt. found: Sample A, 407; Sample B, 408

$$[\alpha]_D^{26} = \frac{+0.18° \times 5}{2 \times 0.0753} = +5.97°\ \text{(in pyridine)}$$

TABLE X

A. Methoxycerebronic Acid

Fraction No.	Yield from 1 gm. ester	Melting point	Solidification point	Molecular weight	$[\alpha]_D^t$
	gm.	*°C.*	*°C.*		*degrees*
1	0.70	74–76	64–65	408	+17 54
				407	
2	0 85	74–76	64–65	411	+18 85
				411	
3	0.80	74–76	67–68	411	+19 14
				409	
4	0.75	74–76	67–68	412	+20 43
				413	
Calculated molecular weight for $C_{24}H_{47}(OCH_3)$-COOH.....				398	
Calculated molecular weight for $C_{25}H_{49}(OCH_3)$-COOH..				412	

B. Data for Determination of Molecular Weight and Specific Rotation

Fraction No	Molecular determination		Specific rotation		
	Substance	0 1 N NaOH	Substance in 5 cc pyridine	α_D, 2 dm. tube	Temperature
	gm.	*cc.*	*gm.*	*degrees*	*°C.*
1	0 0970	2 38	0 0513	+0 36	26
	0 1124	2 77			
2	0 0974	2 37	0 0610	+0 46	26
	0 1184	2 88			
3	0 1314	3 20	0 0653	+0 50	26
	0 1065	2 56			
4	0.1539	3 73	0 6850	+0 56	26
	0 1610	3 90			

C. Analytical Data

Fraction No	Substance	CO_2	H_2O	C	H	Substance	AgI	OMe
	mg.	*mg.*	*mg.*	*per cent*	*per cent*	*mg.*	*mg.*	*per cent*
1	4 300	11.810	4 800	74.89	12.25	6.150	3 350	7.19
2	3 750	10 434	4 300	75 87	12.83	6 980	4 050	7.66
3	3.534	9.805	3 990	75.66	12.63	7.621	4 560	7 89
4	4.301	11 925	4 970	75 60	12 93	9 490	5 280	7 34
$C_{26}H_{52}O_3$ calculated....				75.65	12.70			7.52

Methoxycerebronic Acid.   Sample A—1 gm. portions of each of the four fractions of methyl methoxycerebronate (Table VIII) were dissolved in 50 cc. of hot methyl alcohol, and then 3 cc. of 5 N sodium hydroxide were added.   The mixture was saponified by boiling on the steam bath for about 10 hours.   The sodium salt was decomposed with concentrated hydrochloric acid, and the acid extracted with ether.   After evaporating off the ether, the acid was recrystallized from petrolic ether.   The properties are shown in Table X.

Sample B.   Lead Salt of Methoxycerebronic Acid—To a solution of 4 gm. of methoxycerebronic acid in 100 cc. of hot methyl alcohol was added gradually a solution of 3.6 gm. of lead acetate in 30 cc. of hot methyl alcohol.   A white precipitate formed immediately. When all the acetate solution had been added, a few drops of ammonium hydroxide were added to complete the precipitation. The mixture was then filtered and washed with alcohol.   The yield of lead salt was about 5.5 gm.   It melted at 74° and resolidified at 71–72°.

The lead salt was suspended in benzene and a current of hydrogen sulfide was passed through until all the lead was precipitated. It was then filtered, the filtrate evaporated, and the acid recrystallized from petrolic ether.   The yield was 3.5 gm.   Melting point, 75–77°; solidification point, 64–65°.

Sample 1—0.1402 gm. substance required 3.41 cc. 0.1 N NaOH
    "   *2*—0.1466 "   "   "   3.55 " 0.1 "   "
$C_{26}H_{52}O_3$.   Mol. wt. calculated, 412.4; found, Sample 1, 411, Sample 2, 413

$$[\alpha]_D^{20} = \frac{+0.48° \times 5}{2 \times 0.0590} = +20.34°$$

Methyl and Ethyl Esters of Cerebronic Acid—The methyl and ethyl esters of cerebronic acid were prepared by refluxing separately the solutions of 5 gm. of *d*-cerebronic acid in 250 cc. of absolute methyl and ethyl alcohol containing 15 cc. of concentrated sulfuric acid respectively for 6 hours.   On cooling to 5°, the esters crystallized.   They were filtered separately, and washed and recrystallized from 100 cc. of the respective alcohol.   The methyl ester melted at 64° and resolidified at 61–62°.   The ethyl ester melted at 64–65° and resolidified at 58°.

They were recrystallized separately from 75 cc. of petrolic ether (60–70°), and the melting and resolidification points of both esters remained the same.

OXIDATION AND DERIVATIVES OF *dl*-α-HYDROXY-STEARIC ACID

By P. A. LEVENE and P. S. YANG

(From the Laboratories of The Rockefeller Institute for Medical Research, New York)

This study of the derivatives and of the oxidation products of α-hydroxystearic acid was undertaken as a control to the results obtained on the oxidation products of cerebronic acid.[1] Two points were particularly under investigation. First, is it possible to obtain a pure α-hydroxystearic acid by esterification of the crude material with subsequent hydrolysis of the ester by the method introduced by Thierfelder[2]—allowing to stand in the cold with an alcoholic solution of sodium hydroxide for 15 minutes only? Second, does the pure α-hydroxystearic acid on oxidation with potassium permanganate under the conditions observed in the oxidation of cerebronic acid yield fatty acids lower than margaric acid? Taylor and Levene reported in 1928[3] the results of oxidation of α-hydroxylignoceric acid into α-tricosanic acid only. The present experiments on α-hydroxystearic acid were performed under conditions approaching more nearly those employed in the case of purification and oxidation of cerebronic acid reported in the preceding paper by Levene and Yang.[1]

The results of the present investigation were the following. The separation of the α-hydroxystearic acid from the impurities was accomplished without difficulty and the acid obtained in this manner had the molecular weight as required by theory, namely 300. Thus, it is shown that *the process of esterification with subsequent saponification and liberation of the free acid from the soap in the case of the higher α-hydroxy fatty acids does not lead to lactide formation.*

[1] Levene, P. A., and Yang, P. S., *J. Biol. Chem.*, **102,** 541 (1933).

[2] Thierfelder, H., *Z. physiol. Chem.*, **43,** 21 (1904).

[3] Taylor, F. A., and Levene, P. A., *J. Biol. Chem.*, **80,** 609 (1928).

The oxidation of α-hydroxystearic acid led to the formation of margaric acid only. The oxidation product was esterified and the esters fractionally distilled into three fractions. The acids obtained from all three fractions had the melting point and the molecular weight required by theory for margaric acid.

Purification of dl-α-Hydroxystearic Acid—The crude *dl-α*-hydroxystearic acid prepared from *dl*-bromostearic acid was purified by repeated esterification and saponification in the cold as in the case of *d*-cerebronic acid.[1] After four operations of esterification and saponification, the acid obtained was crystalline and had a molecular weight of 300 (0.2091 gm. required 6.96 cc. of 0.1 N NaOH). It melted at 84–86°, and resolidified at 76°.

This acid was recrystallized from acetone and 116 gm. of crystalline acid were thus obtained having a melting point of 84–86° and resolidification point of 77–78°. The substance had the following composition.

4.265 mg. substance: 11.245 mg. CO_2 and 11.96 mg. H_2O
0.2024 gm. required 6.76 cc. 0.1 N NaOH
$C_{18}H_{36}O_3$. Calculated. C 71 93, H 12.08, mol. wt. 300.3
Found. " 71.89, " 11.96, " " 299.4

Oxidation of dl-α-Hydroxystearic Acid—The oxidation of *dl-α*-hydroxystearic acid was carried out as for the oxidation of *d*-cerebronic acid.[1] 50 gm. of the acid were oxidized in three lots. The amount of potassium permanganate was calculated on the basis of oxidizing 2 carbon atoms with 25 per cent excess (19.6 gm. of potassium permanganate for each 10 gm. of the acid).

The combined crude oxidation product was recrystallized from 300 cc. of hot acetone. It gave a yield of 27 gm. of crystalline acid having a melting point of 54–56° and resolidification point of 53°. The molecular weight was found to be 269 (0.1503 gm. required 5.59 cc. of 0.1 N NaOH).

The mother liquor was concentrated and a second crop of 7 gm. was obtained. It melted at 50–51° and resolidified at 48°. Its molecular weight was 261 (0.1382 gm. required 5.30 cc. of 0.1 N NaOH).

Purification of the Oxidation Product of dl-α-Hydroxystearic Acid—

The first fraction (27 gm.) of the recrystallized oxidation product of dl-α-hydroxystearic acid was purified by esterification and saponification in the cold. After the second operation, the acid obtained melted at 56–58° and resolidified at 54°, and had a molecular weight of 270.5 (0.2026 gm. required 7.49 cc. of 0.1 N NaOH).

It was then esterified with methyl alcohol and distilled, leaving a residue of about 6 gm. The residue was converted into the free acid which melted at 58–59° and resolidified at 55–56°. It had

TABLE I

Fractions of Oxidation Product of dl-α-Hydroxystearic Acid

Fraction No.	Yield of acid	Melting point	Solidification point	Molecular weight determination		
				Molecular weight	Substance	0.1 N NaOH
	gm.	*°C.*	*°C.*		*gm.*	*cc.*
1	6	58–59	55–56	269	0 1467	5 45
				269	0.1177	4 37
2	3	58–59	55–56	270	0 1166	4 31
				271	0 1148	4 24
3	3	58–59	55–56	273	0 1323	4 84
				275	0 1300	4.73

Analytical Data

Fraction No.	Substance	CO_2	H_2O	C	H	Calculated percentage composition
	mg.	*mg.*	*mg.*	*per cent*	*per cent*	
1	3.495	9 650	3 982	75 30	12 71	$C_{17}H_{34}O_2$ (270.27)
2	3 215	8 866	3 655	75 20	12 72	C = 75 60; H = 12 60
3	3 635	10.010	4 010	75 09	12 34	$C_{16}H_{32}O_2$ (256.26) C = 74 92; H = 12 19

a molecular weight of 286 (0.1476 gm. required 5.16 cc. of 0 1 N NaOH).

The distilled fraction was refractionated into three fractions, and each fraction was converted into the free acid. The properties are tabulated in Table I.

The second crop obtained from the mother liquor (7 gm.), together with 3 gm. obtained from the mother liquor of the first fraction, was esterified and saponified in the cold separately The yield of the free acid was 8 gm. It melted at 52–54° and resolidi-

fied at 49°. The acid was then converted into the methyl ester, and the ester distilled at 140–155° at 0.2 mm. (bath temperature 170–180°). The ester was converted into the free acid having a melting point of 53–54°; solidification point 52°. The substance had the following composition.

3.695 mg. substance: 10 250 mg. CO_2 and 4 190 mg. H_2O
0 1074 gm. required 3 90 cc. 0 1 N NaOH
$C_{17}H_{34}O_2$. Calculated. C 75 60, H 12 60, mol. wt. 270 27
Found.   " 75 64, " 12 52, "   "   271

Methyl Ester of dl-α-Hydroxystearic Acid—10 gm. of the *dl-α*-hydroxystearic acid were dissolved in 100 cc. of methyl alcohol containing 3 cc. of concentrated sulfuric acid, and the solution was refluxed for 4 hours on a steam bath. On cooling to 5° the ester crystallized and was filtered off. It was recrystallized twice from 100 cc. portions of methyl alcohol. The yield was 10.5 gm. It melted at 64–66°, and did not resolidify when it was cooled down to 50°. The substance had the following composition.

3.826 mg. substance: 10.140 mg. CO_2 and 4 135 mg. H_2O
$C_{19}H_{38}O_3$. Calculated. C 72 54, H 12 18
(314 3)   Found.   " 72 27, " 12 09

dl-α-Hydroxystearylhydrazide—10 gm. of methyl *dl-α*-hydroxystearate and 5 gm. of hydrazine hydrate were placed in a 250 cc. round bottomed flask fitted with an air condenser, and kept at melting temperature over a free flame during 5 hours, considerable foaming occurring. The melted mixture was then heated on a steam bath overnight, after which 50 cc. of alcohol were added. The solution was cooled, filtered, and the product recrystallized three times with 200 cc. portions of absolute alcohol. The yield of the recrystallized hydrazide was 8 gm. It melted at 136–137° and resolidified at 130–131°. The substance had the following composition.

4.200 mg. substance: 10.655 mg. CO_2 and 4 535 mg. H_2O
4.590 "   "   : 0 360 cc. N_2 (at 23.56° and 759 mm.)
$C_{18}H_{38}O_2N_2$. Calculated. C 68.74, H 12.18, N 8.92
Found.   " 69 17, " 12.08, " 9 02

Methyl dl-α-Methoxystearate—27 gm. of methyl iodide were added to a solution of 10 gm. of methyl *dl-α*-hydroxystearate in

25 cc. of hot acetone and the solution was treated with 22 gm. of silver oxide in small portions at 30 minute intervals. The reaction was carried out at 40-50°,[4] during 5 hours, with continuous mechanical stirring.

The silver salts were then extracted five or six times with hot acetone. After evaporation of acetone the methylated product remained in the form of an oil. It was then distilled, giving three fractions the properties of which are recorded in Table II.

dl-α-Methoxystearic Acid—1 gm. of Fraction 1 (Table II) and 2 gm. of Fraction 2 were dissolved in about 50 cc. of methyl

TABLE II

Methyl dl-α-Methoxystearate

Fraction No.	Temperature	Bath temperature	Pressure	Weight	Melting point
	°C.	°C.	mm.	gm.	°C.
1	135-148	160-170	0 25	3 2	37-38
2	138-148	170-185	0 20	6.2	37-38
3	138-148	170-185	0 20	1 5	39-40

Analytical Data

Fraction No.	Sub-stance	CO_2	H_2O	C	H	Sub-stance	AgI	OMe
	mg.	mg.	mg	per cent	per cent	mg.	mg.	per cent
1	4 250	11 450	4 715	73 47	12 41	9 255	13 300	18.96
2	4 250	11 400	4 675	72.50	12 30	4 801	6 935	19 00
$C_{20}H_{40}O_3$ (328 3) calculated. ...				73 10	12 27			18.88

alcohol and separately saponified with 5 cc. and 10 cc. of 5 N NaOH respectively, by refluxing on a steam bath overnight. The resulting sodium salt of the acid was then converted into the free acid which was recrystallized from petroleum ether. The properties of the two samples of acid are recorded in Table III.

dl-α-Acetylstearic Acid—10 gm. of *dl-α*-hydroxystearic acid were refluxed with 100 cc. of acetyl chloride on the steam bath for about 2 hours. The excess acetyl chloride was then removed by separation on the steam bath, a little water added, and the solution

[4] Hibbert, H., Tipson, R. S., and Brauns, F., *Canad. J. Research*, **4**, 221 (1931).

further heated on the steam bath. The residue was now dissolved in ether, the ether solution thoroughly washed with water to free it from hydrochloric acid, and then dried over anhydrous sodium sulfate.

After filtering and evaporating off the ether from the filtrate, the crude product was recrystallized several times from 50 cc.

TABLE III

dl-α-Methoxystearic Acid

Substance from Fraction No.	Yield of free acid*	Melting point	Solidification point	Molecular weight determination		
				Molecular weight	Substance	0.1 N NaOH
	gm.	*°C.*	*°C.*		*gm.*	*cc.*
1	0.80	72–73	48–50	313	0.0970	3.10
				312	0 1230	3.94
2	1.80	75–76	54–55	316	0 1825	5.77
				315	0 1870	5.93

Analytical Data

Fraction No.	Substance	CO_2	H_2O	C	H	Substance	AgI	OMe
	mg.	*mg.*	*mg.*	*per cent*	*per cent*	*mg*	*mg.*	*per cent*
1	3.910	10 385	4 210	72 43	12 05	5 170	3 915	10.00
2	4.090	10.914	4 430	72.76	12.12	5 821	4 273	9.68
$C_{19}H_{38}O_3$ (314.3) calculated......				72.54	12.18			9.86

* From 1 and 2 gm. of ester.

portions of petrolic ether (60–70°). The yield of the pure product was 9 gm. It melted at 64–65° and did not solidify when cooled to 50°.

4.070 mg. substance: 10.490 mg. CO_2 and 4.110 mg. H_2O.
0.1464 gm.   "   required 4.24 cc. 0.1 N NaOH
    $C_{20}H_{38}O_4$.   Calculated.   C 70.12, H 11.19, mol. wt. 342.3
          Found.   " 70.28, " 11.30, "   " 345

THE ACTION OF PYRIDINE ON SUGARS

By P. A. LEVENE and DOUGLAS W. HILL*

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According to Danilow and his coworkers[1] glucose is converted to fructose in 30 per cent yield by 4 hours heating in pyridine and in 40 per cent yield by heating in quinoline. Austin and Prusait[2] examined this reaction critically and found that only 1 to 10 per cent of the sugar was converted to ketose, depending on the conditions.

With the object of preparing ketoxylose for comparison with the sugar isolated from the urine of pentosuria patients, xylose was treated in the same way. The reaction was followed by titration of the total sugar present by the Lehmann modification of the Fehling's titration and the aldoses by Willstätter and Schudel's hypoiodite titration[3] as modified by Goebel.[4] We were unable to detect any conversion of xylose to ketoxylose by this method and it was then decided to examine other pentoses. In order to insure that the conditions of our experiments were correct we also carried out experiments with glucose. According to our titrations, glucose was converted to fructose in a yield of only 11 per cent and in that respect we are in agreement with Austin and Prusait. We then resumed our experiments with pentoses other than xylose. Arabinose, we found, behaved like xylose but both lyxose and ribose were converted to ketoses, the former to the extent of 11 per cent after 4 hours and the latter to the extent of 8 per cent.

* Commonwealth Fund Fellow.

[1] Danilow, S., Venus-Danilowa, E., and Schantarowitsch, P., *Ber. chem. Ges.*, **63**, 2269 (1930).

[2] Austin, W. C., and Prusait, W., *J. Biol. Chem.*, **97**, lxxx (1932).

[3] Willstätter, R., and Schudel, G., *Ber. chem. Ges.*, **51**, 780 (1918).

[4] Goebel, W. F., *J. Biol. Chem.*, **72**, 801 (1927).

The differences between the pentoses appears to be a further example of the abnormal behavior of the *cis* 2:3 sugars. Both lyxose and ribose are more sensitive to alkali than xylose and arabinose, as was indicated by the low titration figures recorded for these sugars by Levene, Raymond, and Dillon.[5] They found that by their normal procedure the hypoiodite titration of lyxose and ribose gave an average of only 74.8 per cent of the theoretical, and it can be shown that this abnormality is entirely due to the sensitivity of the *cis* 2:3 sugars to alkali.

It was therefore thought that other aldoses having the 2:3 hydroxyls in the *cis* position might show a greater tendency towards rearrangement into the corresponding keto sugars than the corresponding aldoses having the 2:3 hydroxyls in the *trans* position. To test this possibility it was decided to examine *d*-mannose and *l*-rhamnose. Since glucose, which is a *trans* 2:3 sugar exhibits a conversion to fructose it might be expected that mannose, the corresponding *cis* 2:3 hexose, would be converted to ketose to a greater extent. This was actually found to be the case since the product from the treatment of *d*-mannose by 4 hours heating in pyridine contained more than 30 per cent of ketose. Rhamnose, which is also a *cis* 2:3 sugar, was converted to ketose to the extent of 16 per cent.

Polarimetric observations indicated that the reaction proceeds faster than was anticipated and this was definitely demonstrated in the case of *l*-rhamnose by titrating solutions heated at 50° and 100° for 0.5 hour and 3 hours at each temperature. No increase in keto sugar was found in the 3 hour samples over the 0.5 hour samples. Definite equilibria are set up at these temperatures therefore, with, in the case of rhamnose, 5.5 per cent and 11.5 per cent of ketose present respectively and, in the case of mannose, 6.5 per cent and 15.5 per cent respectively. It was shown that a true equilibrium was set up by taking solutions heated at 100° and continuing the heating at 50°. The back reaction was slow but in 2 hours the equilibrium approached that of solutions heated only at 50°. With this in mind we also heated *d*-fructose in pyridine for 4 hours and again found that the back reaction was slow but

[5] Levene, P. A., Raymond, A. L., and Dillon, R. T., *J. Biol. Chem.*, **95**, 699 (1932).

definite since after that time only 56 per cent of the total sugar was present as ketose. Of the total, 44 per cent had therefore been converted to aldose. According to Danilow, who, as previously pointed out, claimed a higher percentage of fructose from glucose than we were able to find, 83.3 per cent of the total sugar remains as ketose after 4 hours.

Mechanism of Reaction—Inasmuch as the mutual rearrangement of the α and β forms of sugars into the equilibrium form is influenced both by solvent and temperature, the possibility was envisaged that in pyridine at high temperatures the predominance of the form which is conducive to the rearrangement of the aldo to the keto sugar is established. It has been suggested by Lobry de Bruyn and van Ekenstein[6] that the formation of a 1:2 ethylene oxidic ring form is the stage preceding the final reaction. In the case of *d*-mannose the β form which is the 1:2 *cis* form should be the more favorable for the formation of an ethylenic oxide. Since it was shown by Haworth and Hirst[7] that in the case of a *cis* 2:3 sugar, the proportion of the 1:2 *cis* form varies with the solvent, being, for example, higher in water than in alcohol, it was thought that in pyridine solution the proportion of the 1:2 *cis* form might be still higher. It was found, however, that the proportion of the 1:2 *cis* form of mannose is lower in pyridine than in water. Thus, both the α and β forms of mannose on standing in pyridine solution reached an equilibrium rotation of $[\alpha]_D^{30} = +48.09°$. Since the specific rotation of the α-mannose was $[\alpha]_D^{30} = +53.87°$ and of the β-mannose was $[\alpha]_D^{30} = +14.74°$, it follows that the percentage of β sugar, which in this case is the *cis* 1:2 was 15.0. The percentage of the *cis* 1:2 sugar in water is 38.1. There is therefore no reason to believe that there is a higher percentage of *cis* 1:2 sugar present in the pyridine solution or, following from this, that the formation of ketose is dependent on a high *cis* 1:2 sugar content in the solution.

The above mentioned ethylene oxide ring formation as an intermediate step in the transformation of the aldo into the keto sugar may still be considered in a modified form. The reaction is readily explained by the following mechanism.

[6] Lobry de Bruyn, C. A., and van Ekenstein, W. A. *Rec. trav. chim. Pays-Bas*, **14**, 56, 203 (1895); **15**, 92 (1896).

[7] Haworth, W. N., and Hirst, E. L., *J. Chem. Soc.*, 1226 (1928).

$$
\begin{array}{ccccc}
& \text{CHOH} & & \text{—CHOH} & & \text{CH}_2\text{OH} \\
\text{O}\!\!<\!\!| & & & | & & | \\
& \text{CH} & \longrightarrow & \text{—O—CH} & \longrightarrow & \text{O}\!\!=\!\!\text{C} \\
& | & & | & & | \\
& \text{CHOH} & & \text{CHOH} & & \text{CHOH} \\
& | & & | & & | \\
& \text{I} & & \text{II} & & \text{III}
\end{array}
$$
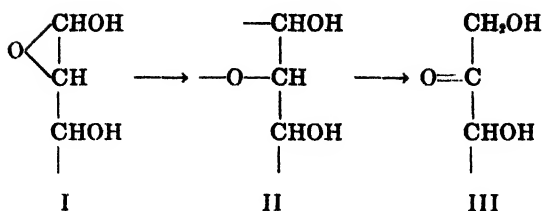
The form (I) may be derived either through the formation of

$$\text{HC}\overset{\text{HO}\quad\text{OH}}{\diagdown\diagup}\text{CHOH—CHOH—}$$

the hydrated form HC—CHOH—CHOH— from the cyclic
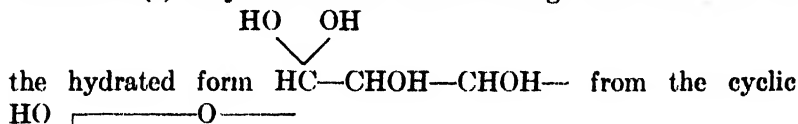
$$\text{HC}\overset{\text{HO}\quad\diagup\!\!\text{—————O————}}{\diagdown}\text{CHOH—CHOH—},$$

HC—CHOH—CHOH—, or from the shifting of the amylene ox-
idic to the ethylene oxidic ring. In anhydrous pyridine solution the
formation of the hydrated form should not take place, whereas the
shift of the ring structure, perhaps through the preliminary forma-
tion of a pyridine addition complex on the oxygen atom engaged in
the ring formation, can easily occur. Whether or not an addition
product is actually formed has not as yet been established. The
fact that saccharinic acids are not formed in the course of the
reaction of anhydrous pyridine on aldoses would indicate that in
the presence of pyridine the ethylene oxide ring opens up as in
Formula II, while for the formation of saccharinic acids it should
open up as in Formula V.

$$
\begin{array}{ccccc}
& \text{CHOH} & & \text{—O—CH—OH} & & \text{O}\!\!=\!\!\text{C—OH} \\
\text{C}\!\!<\!\!| & & & | & & | \\
& \text{CH} & \longrightarrow & \text{—CH} & \longrightarrow & \text{CH}_2 \\
& | & & | & & | \\
& \text{CHOH} & & \text{CHOH} & & \text{CHOH} \\
& | & & | & & | \\
& \text{IV} & & \text{V} & & \text{VI}
\end{array}
$$
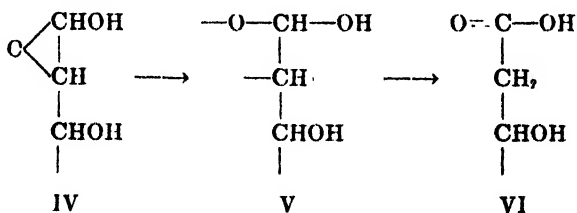
Apparently in the presence of water both types of opening of the
ring take place, for Danilow records the formation of saccharinic
acids in aqueous pyridine solution. The directive influence of
hydrion concentration on the manner of opening of ethylene oxides
has been previously observed by Levene and Walti.[8]

[8] Levene, P. A., and Walti, A., *J. Biol. Chem.*, **73**, 263 (1927).

Conversion of Aldoses to Ketoses in Pyridine—The general procedure was as follows: Solutions of the sugar in 8 times its weight of dry pyridine were gently boiled under a reflux condenser for varying periods.   The pyridine was then removed under reduced pressure and about 50 cc. of water added to the residue and the distillation repeated.   The pyridine was completely removed by two or three such treatments.   The final residue was taken up in

TABLE I

Conversion of Aldoses to Ketoses in Pyridine

Sugar	Solvent	Time	Total sugar	Aldose	Ketose
		hrs.	*gm.*	*gm.*	*per cent*
d-Ribose	Pyridine	4	2.64		
			2 64	2 45	8 0
d-Arabinose	"	4	4 50	4 95	
		8	4 55	4 88	
	Pyridine + 0.5 cc. H_2O	8	4 30	4 62	
d-Xylose	Pyridine	2	5 66		
			5 72	6 00	
		4	5 42		
			5 36	5 70	
		6	5 48	5 55	
	Pyridine + 0 5 cc. H_2O	6	3 85	4 03	
d-Lyxose	Pyridine	4	4 90	4.33	11 1
			4.90	4 34	11 2
l-Rhamnose	"	4	1 42	1 21	14.8
d-Glucose	"	4	5 81	5.13	11.7
d-Mannose	"	4	3 06	2.00	34.6

water, the solution made up to a known volume and the total sugar and aldose present estimated by the Rupp-Lehmann titration and the Goebel modification[4] of the hypoiodite titration[3] respectively. In some instances water was added to the solution and these cases are shown in Table I.   From the values found by the titrations the percentage of ketose formed was obtained by difference.   In order to ascertain the reliability of the method a preliminary experiment was carried out on glucose in exactly the manner described above and for a further comparison of the influence of the *cis* 2:3 hy-

droxyls, *d*-mannose and *l*-rhamnose were also examined. The data for all these sugars are included in Table I.

Standardization of Hypoiodite Titration—The hypoiodite titration was standardized on four of the sugars used. Goebel's modification was used throughout. The results are given in Table II.

Polarimetric Examination of l-Rhamnose in Pyridine—Sealed tubes each containing 5 cc. of a solution of *l*-rhamnose in dry pyridine were kept at room temperature or heated at 50° or at 100° for varying periods and examined polarimetrically in a 2 dm. tube. Those solutions which were heated were removed from the constant temperature bath and plunged into cold water before the tubes were opened and then allowed to come to room temperature before measuring the rotation. The specific rotations are given in Table III.

TABLE II

Hypoiodite Titration of Sugars

Sugar	Sugar present	Sugar found	Percentage sugar found
	mg.	*mg*	*mg.*
d-Ribose	37 54	37.52	99.96
d-Xylose . . .	50 37	50 28	99 62
d-Lyxose.	43 04	42 78	99 40
l-Rhamnose . .	48.40	47 59	98 33

A series of observations was also made on a solution which had been heated to 50° after first being allowed to come to equilibrium at room temperature.

To show the reversibility of the reaction, two sealed tubes containing a pyridine solution of *l*-rhamnose were heated at 100° for 2 hours. One was opened and the rotation observed and compared with that of a similar solution heated at 50° for the same period. The respective specific rotations were $[\alpha]_D^{30} = -24.30°$ and $[\alpha]_D^{30} = -27.70°$. The second tube, heated at 100°, was transferred to a bath at 50° in which it was kept for a further 2 hours. On examination it then had $[\alpha]_D^{30} = -26.97°$, indicating a slow reversibility of the reaction.

Conversion of l-Rhamnose to Ketose at Different Temperatures. Experiment A—10 cc. of a solution of *l*-rhamnose equilibrated at

room temperature in pyridine were evaporated to dryness at room temperature under reduced pressure. Water was added and the distillation repeated. This operation was repeated once more.

TABLE III

Polarimetric Examination of l-Rhamnose in Pyridine

Time	Room temperature; $c = 0.06177$ gm. per cc ; $t = 30°$; $l = 2$		$50°$; $c = 0.05802$ gm. per cc ; $t = 28°$; $l = 2$		$100°$; $c = 0.05802$ gm. per cc.; $t = 28°$; $l = 2$		$50°$ after equilibrium at room temperature; $c = 0.06177$ gm per cc ; $t = 28°$, $l = 2$	
	α	$[\alpha]_D^{30}$	α	$[\alpha]_D^{24}$	α	$[\alpha]_D^{28}$	α	$[\alpha]_D^{28}$
Initial......	-4.26	-34.49	-4.23	-36.46	-4.23	-36.46	-3.58	-28.99
10 min.							-3.45	-27.94
15 "			-3.36	-28.96	-2.72	-23.45		
20 " . ..							-3.34	-27.05
0.5 hr ..			-3.23	-27.84	-2.60	-22.42	-3.31	-26.74
0.75 " ..			-3.20	-27.58	-2.64	-22.76	-3.29	-26.65
1.00 " ...	-3.79	-30.69	-3.21	-27.67	-2.65	-22.84		
1.25 hrs....			-3.17	-27.33				
1.5 "			-3.20	-27.58	-2.64	-22.76		
2.5 " ...					-2.63	-22.68		
4.0 " ...	-3.69	-29.88						
5.5 " ...	-3.51	-28.43						
22 "	-3.57	-28.92						
4 days....	-3.58	-28.99						

TABLE IV

Ketose Formation from l-Rhamnose at Different Temperatures

Temperature of equilibrium	Time of heating	Total sugar	Aldose	Ketose
°C.	hrs.	mg.	mg.	per cent
Room temperature		563	550	0.5
50	0.5	550	517	6.0
50	3.0	541	512	5.4
100	0.5	563	500	11.2
100	3.0	550	484	12.0

The final residue was dissolved in water and the solution made up to 100 cc. The total sugar and aldose present were then estimated in the usual way.

Experiment B—Two similar solutions equilibrated at 50° in sealed tubes for 0.5 hour and 3 hours respectively were treated in the same way except that the solvent was removed at 50° under reduced pressure.

Experiment C—Two further solutions equilibrated at 100° in sealed tubes for 0.5 hour and 3 hours respectively were treated similarly.

TABLE V

Polarimetric Examination of d-Mannose in Pyridine

Time	α-d-Mannose at room temperature; $c = 0.03202$ gm. per cc.; $t = 30°$; $l = 2$		β-d-Mannose at room temperature; $c = 0.03358$ gm. per cc.; $t = 30°$; $l = 2$		Equilibrium mixture at 50°; $c = 0.03202$ gm. per cc.; $t = 30°$; $l = 2$		Equilibrium mixture at 100°; $c = 0.03202$ gm. per cc.; $t = 30°$; $l = 2$	
	α	$[\alpha]_D^{30}$	α	$[\alpha]_D^{30}$	α	$[\alpha]_D^{30}$	α	$[\alpha]_D^{30}$
Initial	3.45	53.87	0.99	14.74	3.08	48 09	3.08	48 09
10 min.....			1.32	19.65				
15 "					2.84	44 34	2.59	40.43
0.5 hr...	3.29	51.37	2.17	32 31	2 80	43 72	2 60	40.60
0.75 "			2 48	36.93			2.62	40.91
1.00 "	3.15	49.18	2.62	39.01	2 79	43 56	2 64	41.22
1.25 hrs...			2.83	41.95			2.60	40.60
1.5 "	3.10	48.40	2.94	43.77				
1.75 "			3.03	44.90			2.59	40 43
2 00 "	3.07	47 93			2.81	43.89		
2.25 "			3.11	46.31				
3.00 "	3.05	47.62			2.80	43.72	2.59	40.43
3.25 "			3.22	47.95				
20 "	3.08	48.09	3.23	48.09				

The results of these determinations are embodied in Table IV and, as was expected, Experiment A showed a negligible conversion to ketose, indicating that at room temperature the changes observed in rotation are due only to mutarotation. Experiments B and C showed increasing quantities of ketose with rise in temperature but there was no appreciable difference caused by heating for different periods, showing that equilibrium is established rapidly.

Mutarotation of d-Mannose in Pyridine at Room Temperature— Solutions of α- and β-d-mannose in dry pyridine were examined

polarimetrically to determine the percentage of each in the equilibrium mixture at room temperature. Equilibrium was reached in about 4 hours, the specific rotation being $[\alpha]_D^{30} = +48.09°$. The calculated percentages of α- and β-mannose present are therefore α, 85.0 and β, 15.0. The change in rotation is shown in Table V.

Change of Rotation of d-Mannose in Pyridine with Change of Temperature—Experiments were carried out in sealed tubes at 50° and 100° in exactly the same way as described for *l*-rhamnose and similar results were obtained. The specific rotations are shown in Table V.

Conversion of d-Mannose to Ketose at Different Temperatures—Sealed tubes containing an equilibrated solution of *d*-mannose in pyridine were heated at 50° for 2 hours and the total and aldo sugar present estimated in the usual way. Total sugar, 0.6120 gm.; aldose, 0.5724 gm.; ketose formed, 6.5 per cent.

A similar experiment was carried out at 100°. Total sugar, 0.5640 gm.; aldose, 0.4770 gm.; ketose formed, 15.4 per cent.

Conversion of Fructose to Aldose in Pyridine—Approximately 1 gm. of fructose was dissolved in 30 cc. of dry pyridine and the solution gently boiled for 3 hours. The pyridine was removed under reduced pressure and the last traces removed by repeating the distillation after the addition of water. The final residue was dissolved in water and the solution made up to 200 cc. and the total and aldo sugar estimated. Total sugar found, 1.128 gm.; aldose found, 0.495 gm.; ketose, 56.1 per cent; conversion, 43.9 per cent.

A NEW METHOD FOR SEPARATING PRESSOR AND OXYTOCIC SUBSTANCES FROM THE POSTERIOR LOBE OF THE PITUITARY GLAND*

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It is now generally admitted that an extract of the posterior lobe of the pituitary gland can be fractionated so as to yield two preparations which differ quantitatively in their capacities to contract the blood vessels and the uterus.

The most recent work in this field, that of Kamm, Aldrich, Grote, Rowe, and Bugbee (1), represents the most nearly complete separation of the two activities. Of the previous workers, Dudley (2) had obtained the best separation. One of his fractions contained only a trace of uterine activity but *presumably* the bulk of the pressor activity. The work was not followed up, however, and Dudley seems to have abandoned the problem of isolating the active substances, being content with the clear demonstration that separate pressor and oxytocic substances could in all probability be obtained from the posterior lobe.

Pituitary extract influences the secretion of urine, the activity of the alimentary tract, and the size of melanophores as well as the blood vessels and the uterus. The two preparations of Kamm, Aldrich, Grote, Rowe, and Bugbee afford a means of determining whether these additional activities are associated with one or the other or with both of their fractions.

It would be useful to have an alternative method for separating the vascular and oxytocic constituents, not only because of the possibility of obtaining purer substances but in order to discover

whether the intestinal, diuretic-antidiuretic, and melanophore constituents are associated with the same fractions as in the Kamm, Aldrich, Grote, Rowe, and Bugbee preparations.

The purpose of this communication is to describe such a method.

The writer has never tried the method of separation of Kamm, Aldrich, Grote, Rowe, and Bugbee. As a matter of fact, they do not give complete details concerning the preparation of their most active pressor material—80 times more potent than the standard powder; they state that, starting with a product 62.5 times as strong as the standard, the "use of *other solvents,* such as *alcohols,* has raised its potency to 8,000%",[1] *i.e.* to 80 times the standard. Incidentally it may be mentioned that the preparation of the 62.5 material had already necessitated six refractionations of a semipure product and had been obtained only with the loss of a large proportion of the starting material. Moreover, the 62.5 material still contained approximately 10 per cent of oxytocic activity per 100 per cent of pressor activity. The oxytocic activity of the 80 material is not stated. By using a different method, Kamm, Aldrich, Grote, Rowe, and Bugbee obtained a fraction in which the ratio of pressor to oxytocic activity was about 100:4. Four repetitions of the process were necessary to obtain this product and further repetitions did not improve it. Nothing is said concerning the potency of this product in terms of standard powder, but there is reason to believe that it was not so very high.

Compared with the method of Kamm, Aldrich, Grote, Rowe, and Bugbee the procedure to be described is simple. The preparation of both pressor and oxytocic fractions, practically as free from contamination the one with the other as is found in the case of pitressin and pitocin, may be accomplished in a morning's work, when one starts from a concentrate of the gland extract. To reach the preliminary concentrate stage requires more time because of two distillations.

The only step in the procedure to be described which is common to that of Kamm, Aldrich, Grote, Rowe, and Bugbee is the initial extraction with acetic acid, though we have employed 0.5 per cent acid instead of 0.25 per cent. Acid extraction has long been employed. The United States Pharmacopœia of 1916 required

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[1] The italics are mine.

it. Since the subsequent steps are absolutely different, if there are constituents other than the vasoconstricting and oxytocic ones they may conceivably be differently distributed in the final products. Hence a comparative study of the alimentary, melanophore, and diuretic-antidiuretic actions with the fractions to be described and pitressin and pitocin may lead to some decision concerning the existence of independent constituents which affect the gut, melanophores, and the secretion of urine.

Method

Principles—A 0.5 per cent acetic acid extract of the gland is first made. After concentration of the solution, the active constituents may be precipitated, along with inert material, by means of alcohol. More inert material may be removed from the alcoholic precipitate by dissolving the latter and precipitating in a single step with $Fe_2(SO_4)_3$, $Ba(OH)_2$, and colloidal iron. The filtrate, after evaporation to dryness, gives a residue containing the pressor and oxytocic constituents. The active part of the residue is soluble in 96 per cent alcohol. When ethyl acetate is added to the alcoholic solution, the pressor constituent precipitates first but is contaminated by oxytocic material. The addition of more ethyl acetate yields an intermediate fraction also containing both substances, and the addition of still more ethyl acetate gives a precipitate in which the oxytocic substance greatly predominates. The first and last precipitates may be further fractionated by variations of this ethyl acetate treatment, which are given below in detail.

It appears then that when the active substances are dissolved in strong alcohol, the addition of ethyl acetate first precipitates the pressor substance and if more ethyl acetate is added the oxytocic substance is thrown down too. Neither constituent is soluble in pure ethyl acetate, and absolute alcohol appears to be a poor solvent also. It has not been proved whether or not the substances are soluble in absolute alcohol. As soon as one works with absolute alcohol in the open it is no longer absolute, and while one can extract active material from a dry residue, starting with absolute alcohol, one cannot be sure that the result is not due essentially to the presence of water absorbed from the air. Alcohol seems to have little or no selective action.

The solubility of these pituitary substances appears to be affected so much by the presence of contaminants that the time is not yet ripe to discuss their solubilities with profit.   In the preceding outline it is quite obvious that in the first place advantage is taken of their insolubility in alcohol when protein is present, and later use is made of the fact that when less contaminating material is present, they are soluble in alcohol stronger than that in which they were originally precipitated.

Details—I have usually started with posterior lobes representing 5 pounds of fresh pituitary glands preserved in acetone.   The material was not of uniform activity.   The writer had no control over its collection; the glands may not have been removed promptly; moreover, they were kept for an unknown period in the frozen state before the posterior lobes were dissected, minced, and placed in acetone.   Even after being received they were rarely used at once.   Some lots had been in the refrigerator for a year before they were worked up.

The lobes are largely freed from acetone by filtration on a Buchner funnel and are then pressed as dry as possible in a small hand press.   They are immediately placed in a mortar and covered with 0.5 per cent acetic acid.   A quantity of finely broken Pyrex glass is added and the tissue ground.   The contents of the mortar are transferred to a 2 liter beaker, with 0.5 per cent acetic acid to make the total volume about 1 liter; 3 gm. of potassium acetate are added, and the mixture is heated to 90° on an electric hot-plate, while being constantly stirred.   The hot solution is poured into 250 ml. bottles and centrifuged for several minutes.   The supernatant solution is then poured off into a 3 liter distilling flask. The residue in the bottles is washed into the original beaker with 500 ml. of 0.5 per cent acetic acid; 2 gm. of potassium acetate are added, and the mixture is brought to 90°, as before.   The solution is centrifuged and the liquid is added to the contents of the distilling flask.   The solution thus obtained is yellow and clear.   If no electrolyte is used, the solution is turbid.   The extract is concentrated to about 50 ml. by distilling it *in vacuo* (by using an oil pump and immersing the flask in a pail of water kept at 50°).   The addition of about 10 drops of caprylic alcohol before beginning the distillation is necessary to prevent frothing; a current of air during the distillation is also advantageous.   To the 50 ml. of distillation

residue (the contents of the flask are now turbid and potassium acetate has accumulated on the sides) 500 ml. of absolute alcohol are added gradually. At first the potassium acetate dissolves and the solution becomes fairly clear. Then, as more and more alcohol is added, a voluminous precipitate appears. The flask is shaken vigorously and allowed to stand for an hour or so—sometimes overnight. Finally, the precipitate is filtered on a Buchner funnel, with a hard filter paper, and is washed with small quantities of absolute alcohol to remove as much potassium acetate as possible. The funnel is placed in a desiccator over calcium chloride and dried *in vacuo*. The yield amounts to about 3.5 gm. The alcoholic solution contains some active material but probably not more than 10 per cent of the total.

This partially purified preparation is treated as follows. (During the next few steps stirring is constant.) The material is dissolved in 175 ml. of 0.5 per cent sulfuric acid in a liter beaker at room temperature. 4.5 gm. of ferric sulfate (Merck's iron tersulfate) are added and, after several minutes, 175 ml. of saturated barium hydroxide are poured in. After another interval of several minutes, 100 ml. of dialyzed iron (Merck's 5 per cent Fe_2O_3) are added. The precipitate by this time is voluminous. The contents of the beaker are filtered on a Buchner funnel. This process is rather slow, but the filtrate is water-clear. The solution is made acid to Congo red paper as soon as possible, with $2 N H_2SO_4$; 1 to 2 ml. are necessary.[2] The solution may be left in this condition overnight if desired. The next step is the removal of the remaining barium. More $2 N H_2SO_4$ is added in 0.1 ml. quantities. After each addition the whole solution is shaken and 15 ml. are centrifuged. When a drop of acid produces no precipitate in the centrifuged solution, the whole solution is centrifuged and the supernatant liquid is poured into a liter distilling flask. It is distilled *in vacuo*, as in the case of the first distillation, but no caprylic alcohol is necessary. When about 25 to 30 ml. remain in the flask, distillation is stopped and the contents of the flask are transferred to a crystallizing dish for final evaporation to dryness with a fan at room temperature. The residue is small. This residue is dissolved in 4 ml. of water. (A little barium sulfate

[2] At this point we have sometimes combined the solutions from two 5 pound lots of glands, but have not modified the subsequent treatment.

may be present but that does not matter.)   The solution is transferred, with 100 ml. of absolute alcohol, to a centrifuge bottle and is allowed to stand, preferably overnight.   The precipitate is separated by centrifuging.   (If this precipitate is transferred to a crystallizing dish with water and evaporated to dryness with a fan, it is found to be white and crystalline and probably owes its slight activity to the small amount of original solution which it carries along.)   The alcoholic solution is poured into a 250 ml. centrifuge bottle.   If 100 ml. of ethyl acetate are now added, a flocculent precipitate is thrown down.   This contains much pressor and considerable oxytocic substance.   Centrifuging is again resorted to, and the clear solution is poured off into a 500 ml. stoppered cylinder.   The precipitate, which is gelatinous, is transferred to a crystallizing dish with about 25 ml. of water. To the solution in the cylinder 100 ml. more of ethyl acetate are added.   Another, less gelatinous, precipitate is thrown down; it too is centrifuged and transferred to a second crystallizing dish with 25 ml. of water.   It contains pressor and oxytocic substances, both in considerable amounts.   The solution resulting from this second precipitation is returned to the 500 ml. cylinder and 200 ml. of ethyl acetate are added.   A third precipitate, white and not at all gelatinous, is thrown down.   This precipitate contains very little pressor substance but is rich in oxytocic material.   The precipitate is transferred to a third evaporating dish with water. The three precipitates are evaporated to dryness with a fan.   The second precipitate is usually added to the next lot of pituitary glands to be worked up.

The first and third precipitates, representing considerable separation of pressor and oxytocic substances, are further purified as follows.

Purification of Pressor Fraction—The first (pressor fraction) is reprecipitated by being dissolved in 4 ml. of H_2O and adding successively 100 ml. of alcohol and 200 ml. of ethyl acetate.   After centrifuging and pouring off the solution, the precipitate is dissolved in 20 ml. of water and transferred to a 250 ml. centrifuge bottle with 20 ml. of absolute alcohol.   80 ml. of ethyl acetate are added, and the bottle is stoppered and thoroughly shaken. On standing, two layers result—one a very small aqueous layer. If the aqueous layer is not obvious in a few minutes, 5 ml. of

H_2O may be added and the shaking repeated. The turbid mixture is centrifuged to separate the layers completely. I have aimed to have less than 10 ml. of aqueous solution. The large upper layer is removed as completely as possible with a pipette. To the aqueous layer in the bottle 20 ml. of absolute alcohol are added, followed by 80 ml. of ethyl acetate. More water will have to be added to obtain two layers. After shaking and centrifuging, the layers are again separated, the ethyl acetate layer being united with that previously obtained. The evaporation residue from these ethyl acetate layers is best reserved for addition to a future lot of glands. It contains both pressor and oxytocic material. The aqueous layer is transferred to a crystallizing dish and evaporated before a fan. The residue is very potent in pressor activity, with a ratio of pressor to oxytocic activity of about 100:6.

A number of variations of this purification process have been tried. Simple reprecipitation alone is not very effective; the passage of much pressor material into solution along with the oxytocic material diminishes the pressor yield greatly.

By omitting the reprecipitation and relying upon the differential distribution between the aqueous and ethyl acetate layers a pretty good separation may be effected. This step has been carried out five times in the case of one preparation; a pressor fraction resulted which was about as good as may be obtained by the method preferred. However, the loss of pressor material (passage into the organic layer) appears to be larger.

I have tried adding sodium chloride to the aqueous solution before applying the partition procedure, with the hope that the smaller quantity of water necessary to produce a two phase system would prevent the passage of so much pressor substance into the organic phase. This device was not conspicuously successful.

Repeated precipitation of the pressor fraction (first ethyl acetate precipitate), with 200 ml. of ethyl acetate each time instead of 100 ml., has been tried. By doing this it was hoped to diminish the passage of the pressor substance into the solvent, but three successive precipitations showed that pressor substance was being lost to a large extent with each precipitation, and the content of oxytocic substance was not diminished to the degree hoped for.

To what extent the elaboration of the preferred process for the

purification of the pressor fraction would further eliminate the oxytocic substance has not been investigated, because material has been too scarce.

When one sees the flocculent nature of the precipitated pressor substance, one does not wonder much that it contains oxytocic material or that reprecipitation is so disappointing in effecting a separation as is evident from the work of Kamm, Aldrich, Grote, Rowe, and Bugbee and from my own experience.   A method based upon distribution between two liquid phases seems to the writer to be more promising.

The final pressor fraction is a non-crystalline substance, clear and glassy, and slightly brown. Sometimes it may be scaled from the glass readily.   It is exceedingly soluble in water but does not seem to be hygroscopic. The biuret test is positive.   When dry, the substance appears to be quite stable.   A residue on a watch-glass (obtained in determining the solid content of a solution), which had been exposed to sunlight and the air of the laboratory for several weeks, was still extremely potent when tested.

The most potent preparation I have had was 78 times as active as the standard powder.   It was clear and glassy to the eye but under the microscope one could see a few crystals, probably a contaminant.   On the whole, the substance is so homogeneous in appearance that it is hard to imagine that what one sees is only an impurity obscuring the real pressor substance.   I have often had very active preparations containing crystals of one sort or another, but whenever it has been possible to separate the crystalline materials enough to test them, they have proved less active than the amorphous material.   A similar preparation which was 50 times as active as the standard powder was analyzed for me by Dr. Hubert Roth, of the Kaiser Wilhelm Institute for Medical Research in Heidelberg.   It left an ash residue of 12.7 per cent, which was unexpected.   The nitrogen content was 16.3 per cent, the sulfur content 1.89 (both figures on an ash-free basis).   How much of the sulfur is inorganic was not determined, but since sulfuric acid is used in the method of separation, some inorganic sulfur might be expected.   These results are given merely to serve as a basis of comparison for future work.

Purification of the Oxytocic Fraction—To free the oxytocic frac-

tion (third ethyl acetate precipitate) from the pressor contaminant, reprecipitation is resorted to. The evaporation residue is dissolved in 4 ml. of water, and 100 ml. of absolute alcohol are added and 200 ml. of ethyl acetate. A slight precipitate is thrown down. This is removed by centrifuging, and to the clear solution 200 ml. more of ethyl acetate are added. This precipitate also is separated by centrifuging and, after pouring off the supernatant solution, the precipitate is transferred to a crystallizing dish with water and evaporated with the aid of a fan. It contains very little pressor material. The ratio of oxytocic to pressor activity for the residue is about 100:2 or 100:3. The substance is hard to describe but its appearance is rather characteristic. It is not homogeneous. Crystals are present, but there is also a material which fractures when the preparation dries. The substance is colorless and, in the crystallizing dish, looks as though it had solidified while the surface was wrinkled under the influence of the fan. Such a precipitate is about 30 times as active as standard powder. A method of purifying this fraction further will be found in the next section.

On one occasion, after adding the second 100 ml. of ethyl acetate (as called for in the section "Details"), the solution and precipitate were allowed to stand for several hours. During this time, greater precipitation seemed to occur than is usually the case, for, when the final 200 ml. of ethyl acetate were added, very little additional material was thrown down (x). Consequently, the second precipitate was dissolved in 4 ml. of water; 100 ml. of alcohol were added and 200 ml. of ethyl acetate. The resulting precipitate was centrifuged, and 200 ml. of ethyl acetate added to the solution. This time the usual type of precipitate for this stage of the process was obtained. The ethyl acetate evaporation residue from this step was united with the evaporation residue of the ethyl acetate solution marked (x) above (the small precipitate having been removed by centrifuging). The combined residue contained a large proportion of the oxytocic substance and very little of the pressor. It is evidently well to centrifuge the three ethyl acetate precipitates as soon as possible after they are formed.

The distribution of the two substances in the three initial precipitates is approximately as follows:

		Oxytocic per cent	Pressor per cent
1st	precipitate	25	70
2nd	"	25	25
3rd	"	50	5

Pharmacological Evidence for Statements concerning the Activity of the Preparations, Together with Some Additional Observations

Methods of Testing—For determining the oxytocic activity the method given in the United States Pharmacopœia X has been employed. The standard used in assays reported in this paper was No. 2202, supplied by the Food and Drug Administration, United States Department of Agriculture. The solutions made from it were placed in 1 ml. ampules and sterilized. The bath held 100 ml.

The pressor assays were made on chloretonized dogs. I have assumed that the pressor activity, like the oxytocic activity, of U. S. P. standard is constant. Smith and McClosky (3) report that oxytocic and pressor activities parallel each other.

Uterine Constituent—The tracing shown in Fig. 1 enables one to judge the degree of pressor action possessed by the final uterine fraction. The rise of pressure produced by an amount of solution containing 11 oxytocic units is seen to be much less than that produced by 0.5 unit of U. S. P. standard powder. In other words, more than 95 per cent of the pressor constituent has been removed. The third and fourth effects were produced by a different lot of the oxytocic material which had been put in ampules for use in other experiments. 1 ml. contained 10 oxytocic units. Comparison of the effect observed with that caused by 0.5 unit of standard powder shows that in this preparation, too, 95 per cent or more of the pressor constituent had been removed.

If a comparison is made of the pressor actions of 10 unit of an oxytocic concentrate and that of 10 units of a pressor concentrate, the difference is quite striking. This is shown in Fig. 2. One of the uterine preparations used to obtain this tracing was pitocin; the other oxytocic and the pressor preparations were my own material. The diphasic rise of pressure of the pressor preparation
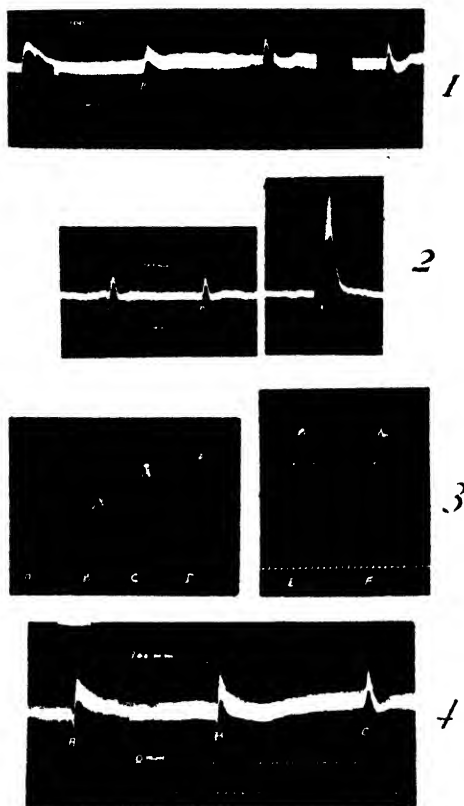
FIGS. 1 TO 4

FIG. 1 *A*, 0.25 mg. of international standard powder; *B*, uterine preparation containing 11 oxytocic units per ml.; *C*, different uterine fraction containing 10 oxytocic units per ml. Time intervals are minutes on all tracings.

FIG. 2. *A*, 10 units of pitocin; *B*, 10 oxytocic units of a uterine fraction; *C*, 10 pressor units of a pressor fraction. 48 minutes elapsed between injections *B* and *C*

FIG. 3. *A*, 0.07 mg. of international standard powder; *B*, 0.00047 mg. of fraction soluble in organic solvent layer; *C*, 0.00071 mg. of the same; *D*, 0.075 mg. of international standard powder; *E*, 0.0031 mg. of fraction soluble in water layer; *F*, 0.08 mg. of international standard powder. The amount used in *C* is about one-fourth of that used in *E* and each quantity is equivalent to about 0.075 mg. of international standard powder. Therefore the material soluble in the organic layer is 4 times as active as that soluble in water (on a weight basis). This comparison shows, too, that the material soluble in the organic layer is about 100 times as active as the international standard powder.

FIG. 4. *A*, 10 oxytocic units from the organic layer; *B*, 10 oxytocic units from the water layer; *C*, 10 oxytocic units as pitocin (1 ml.).

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is due to coronary constriction, which becomes evident when large doses of the pressor preparation are given.

I have not tried hard to concentrate the uterine preparation to the limit. The preparation used in obtaining C in Fig. 1 was only 30 times as active as the standard powder. It is, however, easy to obtain small quantities of material 100 times as active. This may be done as follows: If one dissolves a uterine fraction, prepared as described above, in 10 ml. of water and then adds 10 ml. of absolute alcohol and 80 ml. of ethyl acetate and shakes, two layers result. One then centrifuges and removes the upper ethyl acetate layer with a pipette. On evaporating this layer with a fan a very active, though small, residue is left. The proportion of active substance extracted is from 0.1 to 0.2. The uterine constituent is much less soluble in the organic layer with these proportions of water, alcohol, and ethyl acetate than when the amount of alcohol is doubled.

Others have had more active oxytocic preparations than this. Kamm, Aldrich, Grote, Rowe, and Bugbee state that they have had concentrates 200 times as strong as the standard powder. However, according to a calculation of Abel, based upon the solid content of a pitocin solution, this substance (pitocin) is only 18 times as strong as the standard.

Abel (4) states that his pituitary tartrate was 166 times as active as the standard. It is difficult to reconcile this with his earlier statement that one of his tartrates was 1250 times as active as histamine upon the uterus. This implies that the standard pituitary powder is 7.5 times as strong as histamine acid phosphate. I have been unable to find in the literature any comparison between the standard powder and histamine, but what indirect evidence there is, such as that in a paper by Burn and Dale (5), indicates that histamine produces contraction of the uterus in a higher dilution than does the standard powder. On several occasions I have compared the standard powder with histamine dihydrochloride and found the latter more potent— in one case 4 times as potent and in another 2.5 times. There is, of course, no reason to expect a constant ratio.

Dudley's most active material had both oxytocic and pressor actions. He found it to be about 12 times as potent as histamine. This is not a satisfactory criterion of activity, since it is conceivable

that the uterus may have been relatively insensitive to histamine and highly sensitive to pituitary extract, or *vice versa*.   At the time the work was done, however, this kind of comparison was the best possible.   The statement that the material produced a definite response in a concentration of 1:1,250,000,000 is not very illuminating, since there was no standard of reference in the experiment.   One uterus may be 10 times as sensitive as another.   Consequently, should he have had a very sensitive test object, the concentration given does not indicate as high a degree of activity as at first seems to be the case.

I have never had a uterine preparation free from pressor activity and in consequence have often wondered whether some pressor activity is not inherent in the uterine substance.   The following observation is of interest.   A uterine fraction was treated in the manner described above for increasing the activity of the product. Assay of the two fractions showed that one was 4 times as active as the other (on the basis of weight, not total activity) (Fig.3). When quantities of each containing 10 oxytocic units were injected into a chloretonized dog, the increases in blood pressure were remarkably similar and moreover similar to that produced by 10 units of pitocin.   Fig. 4 shows this.   Since the method of separation indicates that the pressor constituent is less soluble in ethyl acetate than is the uterine substance (the pressor constituent is precipitated by a lower proportion of ethyl acetate), it appears strange that this fractionation caused no separation whatever of the two constituents.   That is, the ratios of the pressor and oxytocic substances in the two fractions are identical.   This may mean that the pressor action of the uterine substance is an inherent property of the oxytocic molecule.

There is another fact that fits in with this possibility.   The pressor curve of the uterine substance is often unlike that of the pressor substance.   In the latter case, with a dose of about 0.5 unit, typical effects are shown in Figs. 1, 5, and 6.   With the uterine fraction, on the other hand, the return of pressure to normal is often, but not always, much steeper.   This is shown in Figs. 1 and 4.   A more striking example of this type of response may be seen in Fig. 5, in which, for the sake of comparison, one may also see a typical response of a pressor preparation.   The oxytocic preparation used in this instance was the same material as was

used in producing the effect (C) in Fig. 1.   Fig. 6 illustrates a striking similarity in respect to this sort of action between one of the writer's preparations and pitocin; normal effects are also shown.   The tracings show numerous similar examples which make it appear that this type of blood pressure response is part and parcel of the action of the oxytocic substance.

This peculiar blood pressure response seems to vary much in different animals.   Often the pressure curve following an injection of the oxytocic substance cannot be told from that resulting from the pressor substance.   On the other hand, *extreme* variation from typical pressure curves is occasionally met with.   In the course of an assay 10 units of pitocin were injected with the surprising result shown in Fig 7.   Several months later the same type of reaction was encountered with one of my own preparations (Fig. 7).   Such effects are troublesome in that they complicate the pressor assay of oxytocic fractions; that is, one cannot be sure that the blood pressure response is not diminished by some depressor mechanism, the influence of which may not always be evident upon the tracing.

The phenomenon in question is not due to histamine.   By means of the Pauly reaction one can detect 0.01 mg. of histamine dihydrochloride contained in 1 ml. of water.   These oxytocic solutions (10 units per ml.) give negative Pauly reactions.   Moreover, these effects cannot be repeated.   A second injection 10 minutes after the first produces a pure pressor response.   Repeated injections of histamine do not result in immunity to histamine action.

Neither is the phenomenon due to a cardiac action; the heart beat as observed in the manometer excursions is not decreased but, as a matter of fact, is often greatly increased.

Pressor Constituent—The elimination of the uterine constituent from the pressor is not accomplished to the same extent with the procedure given as is the elimination of the pressor constituent from the uterine.   Fig. 8 shows a tracing of the assay of a pressor fraction.   The solution used was made by weighing and dissolving the dry substance.   It will be granted that this preparation is about 50 times as active as the standard powder.   On the uterus the tracings (Fig. 9) show that this pressor preparation contained more than 0.5 and less than 0.625 of an oxytocic unit per 10 pressor
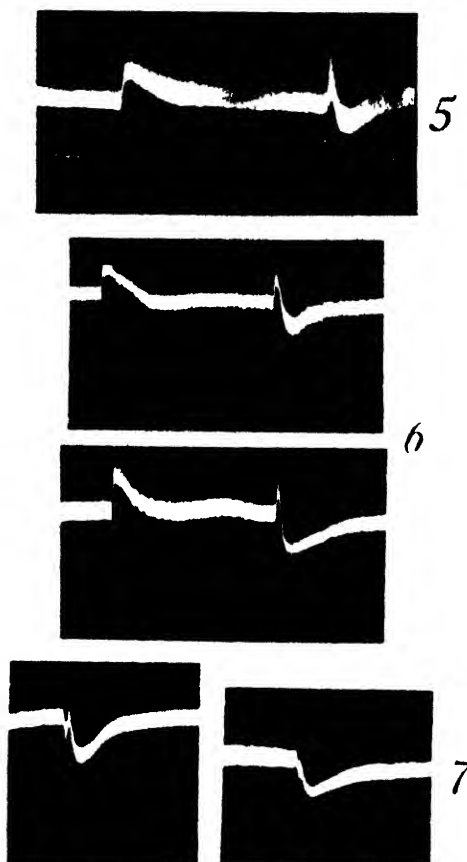
FIGS. 5 TO 7

FIG 5 C, 0.0021 mg. of a pressor fraction 50 times as strong as the international standard, and therefore about one-fifth of a pressor unit; D, 10 oxytocic units of a uterine fraction

FIG. 6. Upper tracing; first effect, 0.25 mg of standard powder; second effect, 10 units of pitocin (C-693807).   Lower tracing; first effect, 0.25 mg. of international standard powder; second effect, 20 oxytocic units of a laboratory preparation.   Horizontal lines are those of 0 mm. of Hg and 100 mm. of Hg

FIG. 7. Left-hand tracing; 10 units of pitocin (C-693807).   Right-hand tracing; 10 oxytocic units of a laboratory preparation (same as was used in Fig. 5).   Horizontal lines are those of 0 mm. of Hg and 100 mm. of Hg.

units.   In other words, approximately 94 per cent of the oxytocic
activity had been removed.   Kamm, Aldrich, Grote, Rowe, and
Bugbee found that their pressor substance contained 0.4 oxytocic
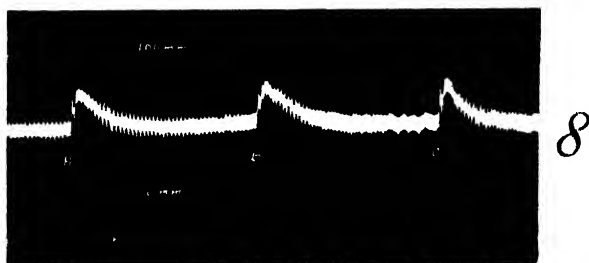
FIGS. 8 TO 10

FIG. 8. *A*, 0 005 mg. of a pressor fraction; *B*, 0.250 mg. of international
standard powder; *C*, same as *A*.

FIG. 9. *A*, 0.025 mg. of international standard powder; *B*, 0 8 of a pressor
unit from a pressor fraction; *C*, 0.020 mg. of international standard powder;
D, does not concern us.   The figure shows that there is less than 0.625 of an
oxytocic unit per 10 pressor units.

FIG. 10. First effect, 0.4 mg. of international standard powder; second
effect, 0.0051 mg. of a pressor preparation.

unit per 10 pressor units after fourteen fractionation treatments. Fig. 10 shows the pressor assay of a preparation about 78 times as active as standard pituitary powder. This is the most active pressor preparation I have had. The ratio of pressor to oxytocic activity was 100:5.

The literature affords no basis for assuming that the substance in question is the pressor compound. Abel has had material 50 times as potent upon the blood pressure as standard pituitary powder and which was, in addition, 50 times as active as the standard powder upon the uterus. If the pressor and oxytocic constituents are assumed to have similar molecular weights, the real pressor substance would have to be at least 100 times as active as the standard powder. Abel also mentions a preparation derived from pitressin which was "conservatively estimated" to be 240 times as active as the standard powder—a figure which he believes to be far below the real value. The potency of my material is brought out by comparing it with adrenalin. I have injected 0.005 mg. of adrenalin from a newly purchased, freshly opened bottle and compared the pressure rise with that produced by 0.005 mg. of my pressor substance, using a chloretonized dog. The rise produced by the pituitary substance was higher and lasted about 40 times as long. This type of comparison is open to the objection, of course, that one is not justified in assuming a constant ratio of activity for adrenalin and pituitary extract.

Kamm, Aldrich, Grote, Rowe, and Bugbee state that they have had material 80 times as active as the standard.

If my material is compared with pitressin on a weight basis, the former is found to be more potent. Abel states that 1 ml. of pitressin (20 units) yields a residue weighing 0.63 mg. On this basis our best material is 4.3 times as active as pitressin. I have determined the residue left by drying 1 ml. of pitressin on a water bath (Abel's method was different) and found it to be 0.34 mg. This would make my material 2.3 times as strong . It is true that pitressin contains chloretone, but this is easily volatile and it would seem that the weights obtained by Abel and myself should represent the actual solid content of pitressin minus the chloretone.

Remark—For general use the author suggests the names "post-lobin-V" and "postlobin-O" for the vascular and oxytocic prepara-

tions, respectively, which are derived from the posterior lobe of the hypophysis.

SUMMARY

A procedure for separating pressor and oxytocic substances from the posterior lobe of the pituitary gland is described.

Inert material is first removed by two successive precipitations, by employing (1) alcohol and (2) barium hydroxide, ferric sulfate, and colloidal iron. The concentrate obtained is dissolved in dilute alcohol and the solution is fractionally precipitated with ethyl acetate. This results in a partial separation. The fractions are further purified by a step which utilizes the different distribution of the substances between the two phases of a water-alcohol-ethyl acetate system, the pressor substance concentrating in the aqueous phase and the oxytocic substance in the ethyl acetate phase.

Some observations upon the chemical properties and pharmacological actions of the fractions are presented.

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GLYCOGEN FORMATION IN THE WHITE RAT AFTER ORAL ADMINISTRATION OF PROPIONIC, BUTYRIC, VALERIC, AND CAPROIC ACIDS*

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Although the question of the conversion of fat to carbohydrate in the animal organism is one that has occupied the attention of physiologists for a long time, it is quite evident from recent reviews on this question that the conclusions drawn from such studies are still quite conflicting. The only unanimity to be found is that the glycerol part of the fat molecule can be and is transformed to glycogen by certain animals. Nothing can be gained by reviewing the voluminous literature on the controversy, because the differences in opinion are based for the most part not on variations in facts but upon their interpretation. Dann (1) has recently presented a very good résumé on this question and the writer recommends that review to those who desire a detailed presentation of this literature. The advent of Cori's method has made it possible to study the question anew and it is the purpose of this paper to report the results obtained by applying this general procedure. It was used to determine whether propionic, butyric, valeric, or caproic acids can form glycogen in the white rat.

The technique employed was similar to that which had previously been used in this laboratory to determine the absorption of and glycogen formation from such substances as amino acids and glycerol (2, 3). All of the experiments were conducted with young male rats weighing from 110 to 160 gm. after a 24 hour fasting period. In all instances known amounts of the acids were administered by means of the stomach tube. In some cases the

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* A preliminary report of a part of this investigation appeared in *Proc. Soc. Exp. Biol. and Med.*, **29**, 100 (1931–32).

acids were given as such, in others they were first half neutralized, and in still others they were introduced in the form of the sodium salt.   The glycogen contents of the livers and the bodies (*i.e.* the glycogen content of the whole animal minus the liver and gastro-intestinal tract) were determined by the method of Pflüger, and the glucose formed by the hydrolysis of the glycogen by the method of Hagedorn-Jensen.   Paired controls were used and these, like the experimental animals, were fasted for 24 hours before being used.   While the animals were not always litter mates, they were nevertheless approximately of the same age and weight and had been upon the same dietary régime.   In order to ascertain whether the substances administered were actually absorbed, an examination was made of the contents of the gastrointestinal tract as soon as possible after the experiments had been completed. This was done by washing out the tract with water, diluting the fluid so obtained to approximate 250 cc., acidifying the mixture with 2 cc. of concentrated sulfuric acid, and distilling until about 175 cc. of distillate had been collected.   The amount of fatty acid in the distillate was then determined by titration with standard alkali.   The validity of this procedure was determined by introducing known amounts of the various acids by stomach tube into fasted animals that were killed immediately.   An examination of the gastrointestinal contents of such animals showed that at least 95 per cent of the acids administered was invariably recovered. This speaks for the reliability of the process.   While no attempts were made to study the relative rate of absorption of the different acids, it was possible to show that all of them were absorbed in such amounts that the failure to find an increase in liver glycogen after the administration of some of them could not be ascribed to a fault in their absorption.

Most of the data obtained are shown in Table I.   For the sake of brevity only the averages are given for the animals into which butyric, valeric, and caproic acids had been introduced.   It is evident from those figures that no increases in the liver glycogen contents took place when those substances were administered. On the other hand it is easily to be seen from Table I that definite increases of liver glycogen did take place when propionic acid was introduced.   More detailed data are therefore presented for those cases.   In some instances the increments were not large, but in

TABLE I

Glycogen Formation after Administration of Propionic, Butyric, Valeric, and Caproic Acids

Form in which administered			Absorption period	Amount introduced	Liver glycogen
			hrs.	mg	per cent
Sodium butyrate	Average of 5 rats		3-9	576–1250	0 06 (0.05–0.07)†
	" " 5 controls*		0	0	0.07 (0.04–0.10)
Sodium valerate	Average of 6 rats		3	200– 800	0.10 (0.08–0.12)
	" " 6 controls		0	0	0.07 (0.06–0.08)
Valeric acid (half neutralized)	" " 6 rats		3	300	0.14 (0 08–0.30)
	" " 6 controls		0	0	0.10 (0 08–0.13)
Valeric acid	" " 3 rats		3	300	0 12 (0 10–0 15)
	" " 3 controls		0	0	0.09 (0 08–0 10)
Sodium caproate	" " 8 rats		3-6	200– 400	0.08 (0 05–0 10)
	" " 8 controls		0	0	0 08 (0.05–0.09)
	Rat No.				
Sodium propionate	9		4	1086	0 15
	11		4	1086	0.15
	12		4	1086	0.12
	15		4	250	0.21
	16		5	250	0 23
Average					0.17
Propionic acid	13		3	500	0 17
	18		4	232	0.26
	19		4	232	0 45
	21		6	250	0.09
	22		6	250	0.13
	23		6	250	0 11
	25		4	200	0 21
	26		4	200	0 26
	27		4	200	0.08
	29		4	200	0.51
	30		4	200	0 16
	35		4	200	0 09
	37		4	200	0 97
	38		4	200	1.37
	41		4	200	0 31
	42		4	200	0 32
	43		4	200	0 12
Average					0 33

TABLE I—*Concluded*

Form in which administered	Rat No.	Absorption period	Amount introduced	Liver glycogen
		hrs.	mg.	per cent
Propionic acid	47	4	200	0.25
(half neutra-	48	4	200	0.13
lized)	49	4	200	0 53
Average...				0.30
" of 16 controls....		0	0	0 06 (0 04–0 09)

* These animals as well as the rest of the controls received no fatty acid.
† The figures in parentheses are the ranges observed for each group.

others as in the cases of Animals 19, 29, 37, 38, 41, 42, and 49 the magnitudes were of such an order to warrant the conclusion that glyconeogenesis had occurred. These increases in liver glycogen cannot be due to a mobilization of body glycogen for the reason that little difference was found to exist between the body glycogen contents of the control rats and those fed propionic acid. The average of the amount of body glycogen in rats into which that acid had been introduced was 0.07 per cent as compared with 0.06 per cent for the controls. Similar likenesses were found to exist between the body glycogen contents of the animals into which the other acids had been administered and those of their controls.

SUMMARY

With use of the Cori technique experiments were conducted with young white male rats to determine the absorption of and glycogen formation from propionic, butyric, valeric, and caproic acids. While an increase in liver glycogen took place when propionic acid was administered, no such an effect was observed when the three other acids were employed. Evidence was obtained to show that the failures in this respect could not be ascribed to a fault in absorption. Determinations of the body glycogen of the rats showed that a mobilization of body glycogen had not taken place.

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A CONVENIENT AND ACCURATE METHOD FOR THE DETERMINATION AND DETECTION OF CARBON MONOXIDE IN BLOOD

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Most of the qualitative tests for carbon monoxide in blood are of little value if less than 10 per cent of the hemoglobin is combined with carbon monoxide. Contrary to the impression that one might obtain from many texts, the ordinary direct vision spectroscope is likewise of little value for the detection of carbon monoxide in blood unless the content of carbon monoxide is relatively high. By the use of a special spectroscope, known as the Hartridge reversion spectroscope, the detection and determination of low concentrations of carbon monoxide in blood can be accomplished. The need of a special, somewhat expensive apparatus will prohibit the use of this method by many laboratories. The value of the ordinary spectroscope as compared to the Hartridge reversion spectroscope has recently been discussed by Frederick (1).

The two simplest methods for the quantitative determination of carbon monoxide in blood, the carmine method of Haldane (2) and the pyrotannic acid method of Sayers and Yant (3), may give fairly accurate results in the hands of those who have a keen color perception, if the bloods contain relatively high concentrations of carbon monoxide. For the lower concentrations of carbon monoxide the value of these methods is questionable. The iodine pentoxide method, introduced by Tervaert (4) and modified by Pilaar (5), enables one to determine small amounts of carbon monoxide with 1 cc. samples of blood. Whether the apparatus and technique are too complicated to be acceptable to the average hospital laboratory remains to be seen. There is no question that the use of the accurate manometric methods of Van Slyke and his asso-

ciates (6–8) for the determination of carbon monoxide in blood has been limited because of the special apparatus and special technique required. The determinations of carbon monoxide by Gettler and Mattice (9) and Hanson and Hastings (10) were made by modifications of the Van Slyke and Neill manometric procedure. As Gettler and Mattice point out, the actual time required for the determination by these methods, if one includes the time required for the deaeration of solutions, is considerably longer than the half hour stated by the original authors.

In the present paper the authors describe a procedure for the determination of carbon monoxide in blood, which requires only a small amount of special apparatus. If one is interested in the qualitative detection of carbon monoxide in blood, only 15 to 30 minutes are required, but 1 to 2 hours are required for duplicate quantitative determinations. Amounts of carbon monoxide representing a 1 per cent saturation of the hemoglobin can be determined.

Principle of Present Method

The blood gases, including carbon monoxide, are released from the blood under reduced pressure by the action of acid ferricyanide solution. The resulting gas mixture is passed into a bulb containing palladium chloride solution. The carbon monoxide reacts with the palladium chloride according to the following equation: $CO + PdCl_2 + H_2O = Pd + CO_2 + 2HCl$. After the above reaction is completed, the excess palladium chloride is separated from the metallic palladium by filtration and the palladium chloride is determined colorimetrically. The addition of potassium iodide in excess of that required to precipitate the palladium chloride as palladous iodide, redissolves the palladous iodide to give a red solution, which in the presence of a protective colloid such as gum ghatti remains perfectly clear for at least 24 hours. The red color is easily matched and fortunately the intensity of the color is almost exactly proportional to the amount of palladium chloride present over a wide range of concentrations. Thus when the color produced from 2 mg. of palladium chloride is made to a volume of 50 cc. and set at 20 mm., 4 mg. and 1 mg. of palladium chloride treated similarly gave readings of 39.8 mm. and 10 mm. In an earlier report one of us (11) used the reduction

of phosphomolybdic acid by the metallic palladium which results from the reaction of carbon monoxide with palladium chloride as a qualitative test for carbon monoxide in blood. From the intensity and shade of the color produced by the addition of phosphomolybdic acid to the metallic palladium, a semiquantitative estimate of the amount of carbon monoxide in the blood could be made. This procedure was abandoned when it was discovered that the color reaction of palladium chloride with potassium iodide could be readily applied to a quantitative determination.

The use of palladium chloride for the determination of carbon monoxide in blood is not new. Von Fodor (12) in 1880 published methods for the determination of carbon monoxide in blood and air. Carbon monoxide was liberated from blood by heating on a steam bath, and passed into a palladium chloride solution by a slow current of air. The metallic palladium resulting from the reaction was removed by filtration, washed, and dissolved in hydrochloric acid. The amount of palladium chloride formed in this way was determined by titration with a standard solution of potassium iodide. The end-point was reached when the addition of potassium iodide produced no further turbidity. In comparison with present methods for blood gases, it is doubtful whether the method of von Fodor can be considered quantitative. There have been numerous methods introduced recently for the determination of carbon monoxide in air, which depend upon the reaction of carbon monoxide with palladium chloride. To our knowledge, however, no recent attempts have been made to apply it to the determination of carbon monoxide in blood.

Reagents

Acid Ferricyanide Solution—This is prepared according to the directions of Van Slyke and Hiller (13). 32 gm. of potassium ferricyanide are dissolved in water and made to a volume of 100 cc. The acid solution is prepared by combining 23 cc. of this solution with 2 cc. of concentrated lactic acid of specific gravity 1.2. This solution is said to keep for 2 months. We have preferred to prepare it in small quantities and renew it at intervals of 2 weeks.

Caprylic alcohol.

Gum Ghatti Solution—5 gm. of gum ghatti are added to 500 cc.

of distilled water and allowed to stand for 24 to 48 hours with occasional shaking. After filtration a clear solution results which appears to keep indefinitely, although it becomes slightly turbid on standing and must be refiltered from time to time.

A *10 per cent solution of aluminum sulfate.*

A *solution of potassium iodide* containing 15 gm. of this salt per 100 cc. of solution. Since this solution develops a yellow tinge on standing, only enough for immediate use is prepared.

Palladium Chloride Solution—500 mg. of palladium chloride (Baker and Company) are placed in a 400 cc. beaker and covered with 150 cc. of distilled water. 2.5 cc. of concentrated hydrochloric acid are added and the mixture is heated until complete solution of the palladium chloride results. After cooling, this solution is transferred to a 500 cc. volumetric flask and made to volume. The palladium chloride salt used in this work was practically dry, since there was little if any loss in weight after drying at 100° for 1 hour. The acid solution of this salt which was prepared to contain 1 mg. of the salt per cc. of solution was analyzed by the standard gravimetric procedure which consists of the precipitation of palladium as the salt of dimethylglyoxime. The analysis indicated that the salt was 99.5 per cent pure. The error introduced by assuming that the salt was 100 per cent pure is negligible and in all of our work such an assumption was made. A palladium chloride solution prepared as directed above remains perfectly clear for long periods if it is kept in tightly stoppered bottles to exclude laboratory dust and air. It is advisable, if large volumes of this solution are made, to store it in a number of small bottles rather than in one large bottle to minimize the deterioration due to exposure to air when samples are removed for use.

Procedure

Preparation of Apparatus—The apparatus required for this determination is shown in Fig. 1. It can be made in a short time by anyone with a little experience in glass-blowing. A is a thick walled test-tube, 250 × 20 mm. (inside diameter), and has a capacity of 50 cc. Reservoir C of the dropping funnel has a capacity of 10 cc. and for convenience should be roughly graduated in 1 cc. steps from 0 to 5 cc. Tube D has an inside diameter of

approximately 3.5 mm. and is set in the rubber stopper so that its opening into the test-tube is slightly higher than the lower surface of the stopper.   A thick walled rubber tubing provided with a Hoffmann pinch-cock is connected to the other opening of tube D. Bulb E should have a capacity of 25 to 30 cc.   Tubes F and I, while not strictly capillary, have an inside diameter of approxi-
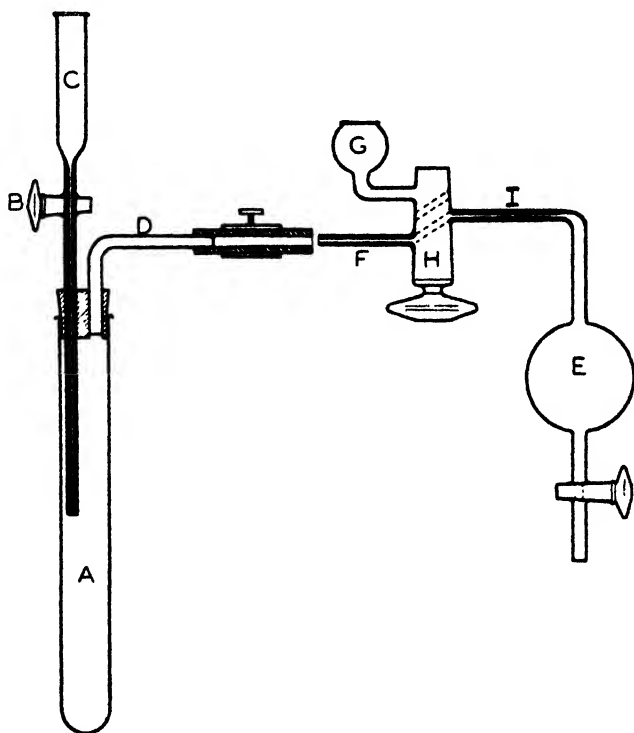
Fig. 1. Apparatus for the determination of carbon monoxide in blood. A, test-tube; B, stop-cock; C, reservoir; D, tube, 3.5 mm.; F and I, glass tubing, 1.5 mm.; G, reservoir; H, stop-cock; E, reservoir.

mately 1.5 mm.   Reservoir G has a capacity of 4 to 5 cc.   The opening through the lower stop-cock of bulb E should not be too small, since particles of metallic palladium must pass through.

Three glass beads are placed in tube A and the 2-hole stopper with the parts indicated in Fig. 1 is tightly fitted into the tube. Stop-cock B is closed and the outlet tube D is connected by the

heavy walled rubber tubing to a water pump and tube A is evacuated. The pressure within the tube should be reduced to such a degree that when the outlet tube is closed by a Hoffmann pinchcock, water admitted through stop-cock B will fill tube A to within 1 or 2 cc. A few trials should be made to find the time required to produce such a reduction in pressure. When tube A has been evacuated sufficiently, the pinch-cock on the tubber tubing is tightly closed and the water pump disconnected. 3 or 4 drops of caprylic alcohol are now placed in cup C, and drawn into the tube by negative pressure.

Treatment of Blood—4 cc. of distilled water are placed in reservoir C, preparatory to the delivery of 2 cc. of blood into tube A. By keeping the tip of the pipette containing the blood at the capillary exit of cup C, and barely opening stop-cock B, the blood may be drawn into the tube ahead of the water. The stop-cock is closed and the pipette is washed once with water drawn from the cup. This washing and the remaining water in the cup are in turn drawn into the reaction tube A. An additional 1 or 2 cc. of water may be necessary to transfer the last traces of blood from the cup and the capillary leading to the tube. The contents of the tube are gently mixed to hemolyze the blood. 0.3 cc. of the acid ferricyanide solution and 1 cc. of distilled water are mixed in cup C and slowly drawn into tube A. The tube is shaken gently, while the ferricyanide is added in order to insure a thorough mixing. 1 or 2 cc. of water are now drawn into the tube to wash the ferricyanide completely into the blood sample. The total volume of distilled water used in the transference of the blood and ferricyanide to the tube should not be greater than 9 cc. when 2 cc. of blood are used. Directions for 1 cc. and 5 cc. portions of blood will be given later in the paper. The contents of the tube are shaken gently for a period of 8 minutes, care being taken that the contents of the tube do not touch the stopper in the region of the outlet tube D. It has been found convenient to start two determinations and shake them simultaneously for the 8 minute period.

Transfer of CO to Palladium Solution—At the end of the 8 minute period, the tubes are set aside while the palladium chloride is measured into bulb E. This bulb is partially evacuated either by suction by mouth or water pump through the tube F extending from stop-cock H. 3 cc. of palladium chloride solution,

accurately measured, are drawn into bulb E by negative pressure from reservoir G. Every trace of palladium chloride in reservoir G and tube I is drawn into the bulb by the addition of 2 to 3 cc. of distilled water. Stop-cock H is turned so as to open along the tube F. This tube is now attached to the water pump and the bulb evacuated. Too great a reduction of the pressure within the bulb may cause the palladium chloride solution to splash toward the capillary I. This is to be avoided since any loss of palladium chloride will give high results. Stop-cock H is now closed and tube F is firmly connected with the rubber tubing attached to outlet tube D. The pinch-cock closing this rubber tubing is now removed and stop-cock H is opened to allow the gas mixture from tube A to pass into bulb E. Tube A and the connecting bulb are now shaken for an additional period of 5 minutes. The gases extracted from the blood are forced into the receiving bulb E by the passage of mercury from cup C into tube A. By properly tipping the tube so as to localize the last few cc. of gas directly beneath the outlet tube D, every trace of the gas may be removed from the tube. In case a small bubble remains trapped at the top of the tube, it should be remembered that its actual size is exaggerated by the vacuum. Moreover the bubble is not all carbon monoxide, since there are also present nitrogen, oxygen, and carbon dioxide both from the blood and from air dissolved in the water and ferricyanide reagent. When the liquid from tube A has been forced by the mercury into the outlet tube D, stop-cock B is almost closed so that the movement of liquid along D toward F is very slow. The liquid is allowed to fill the capillary of stop-cock H but not a trace of the liquid must be allowed to enter the bulb containing the palladium chloride. This can be accomplished without difficulty if stop-cock H is partially closed as the liquid approaches, so that a slight movement will shut it off completely. At the same time it is advisable to turn off stop-cock B. Traces of the blood-ferricyanide mixture which enter the capillary beyond stop-cock H will eventually precipitate some of the palladium chloride and thus give high results.

Determination of Excess Palladium Chloride—Bulb E is now disconnected from the reaction tube A, and a small amount of water (0.5 to 1.0 cc.) is placed in the reservoir G. Stop-cock H is turned so as to fill the capillary along I with distilled water, thus forcing

all of the carbon monoxide into contact with the palladium chloride solution. The bulb containing the palladium chloride is shaken intermittently for a period of 10 minutes. At the end of this period stop-cock H is cautiously opened to allow air to enter, since the contents of the bulb are still under reduced pressure. It is assumed that the determinations are deing made in a laboratory where 5 or 10 cc. of air will contain a negligible amount of carbon monoxide gas. A little water (1 to 2 cc.) is again placed in reservoir G and capillary I is filled with water. The bulb and its contents are set aside with intermittent shaking for a period of 20 minutes. At the end of this time 0.1 cc. of the 10 per cent aluminum sulfate is forced into the bulb, and the contents mixed and allowed to stand for a few minutes. The purpose of the aluminum sulfate solution is to flocculate the colloidal palladium which results from the reaction of carbon monoxide with palladium chloride. For bloods very high in carbon monoxide, it may be necessary to add an additional 0.1 cc. of the aluminum sulfate solution if it is apparent that the first portion has not completely flocculated the colloidal material.

The excess of palladium chloride is now separated from the metallic palladium by filtration into 50 cc. volumetric flasks. Schleicher and Schüll 5.5 cm., No. 589 Blue Ribbon, or Whatman 7 cm., No. 40, paper has proved very satisfactory in our hands. Any filter paper which will not allow the metallic palladium to pass and from which one can quantitatively recover added palladium chloride will be satisfactory. Bulb E is washed thoroughly to remove every trace of palladium chloride. The volume of liquid in the 50 cc. flask, after bulb E and the filter paper have been thoroughly washed, is usually between 25 and 30 cc. If the filter paper is satisfactory, this solution should be perfectly clear. 2 cc. of gum ghatti are added to the solution and mixed. 5 cc. of 15 per cent potassium iodide are now added with shaking. It has been found that small amounts of palladium chloride are adsorbed by the filter paper. To remove these last traces of palladium chloride, the paper is washed twice with 2 cc. portions of the potassium iodide solution and once with a 1 cc. portion. Each washing with potassium iodide solution is followed by small volumes of distilled water. These washings are filtered directly into the colored solution already in the volumetric flask and the contents

of the flask finally made to volume. A small trace of caprylic alcohol added to the flask will minimize the foaming due to the presence of the gum ghatti.

The standard for the colorimetric comparison is prepared by the addition of 2 cc. of the palladium chloride solution, 25 cc. of water, 2 cc. of gum ghatti solution, and 10 cc. of 15 per cent potassium iodide to a 50 cc. volumetric flask. The last solution is added with shaking and the resultant solution made to volume. The color produced by the addition of potassium iodide reaches its maximum intensity in a few minutes and remains constant for at least 24 hours. The standard color is set at 20 mm. Small differences in colorimetric readings alter the final results considerably, especially in the case of bloods containing low concentrations of carbon monoxide. It is, therefore, of especial importance that samples of the standard color be put in both cups of the instrument and that the instrument and mirror be adjusted to the light until equality of color is obtained with both cups set at 20 mm.

Remarks on Details—With the directions which have been given for the use of 2 cc. of blood, concentrations of carbon monoxide hemoglobin representing 5 to 60 per cent saturations of the blood may be accurately determined. For saturations of carbon monoxide up to 10 per cent, such as one might expect to find if studies of chronic poisoning with carbon monoxide were made, 5 cc. of blood are recommended instead of 2 cc. The same procedure is followed, except that 0.75 cc. of the ferricyanide solution is used, and not more than 20 cc. of water are used to transfer the blood and ferricyanide to the reaction tube *A*. The amount of palladium chloride in the receiving bulb may also be decreased to 2 cc. if one is certain that the carbon monoxide saturation is no more than 10 per cent. From Table I it is obvious that for some of the analyses with 5 cc. of blood the concentrations of carbon monoxide were relatively high. In these cases 4 or 5 cc. of palladium chloride solution were used in the receiving bulb *E*. If a determination is started with 2 or 3 cc. of palladium chloride in the receiving bulb and it appears that after the 10 minute period in which the carbon monoxide has reacted with the palladium chloride not enough palladium chloride is present, more may always be added, since the contents of the bulb are still under reduced pressure.

Occasionally one may wish to make carbon monoxide determina-

tions on the blood of small laboratory animals and find it inconvenient to obtain more than 1 cc. of blood. Determinations with 1 cc. of blood may be made by this method provided the saturation of the hemoglobin with carbon monoxide is not less than 10 per cent. For 1 cc. of blood, 0.15 cc. of the ferricyanide solution and not more than 5 cc. of distilled water are used. With 2 mg. of palladium chloride in the bulb E saturations of hemoglobin with carbon monoxide up to 60 per cent can be determined.

Calculation—From the equation of the reaction of carbon monoxide with palladium chloride, it may be calculated that 1 mg. of palladium chloride is reduced by 0.1577 mg. or 0.1261 cc. of carbon monoxide. Since it is generally accepted that 1 gm. of hemoglobin combines with 1.34 cc. of carbon monoxide gas (0° and 760 mm. pressure), 0.0941 gm. of hemoglobin will be required to bind 0.1261 cc. Therefore, each mg. of palladium chloride which is reduced indicates the presence of 0.0941 gm. of hemoglobin bound with carbon monoxide. The excess of palladium chloride over that which reacts with the carbon monoxide is determined colorimetrically. The mg. of palladium chloride reduced by carbon monoxide are obtained by the subtraction of this value from the amount originally placed in bulb E. The mg. of palladium chloride reduced by 100 cc. of the blood are obtained by the multiplication of this last figure by 100, 50, or 20, depending on whether a 1, 2, or 5 cc. sample of blood was used in the analysis. This value is converted to gm. of carbon monoxide hemoglobin per 100 cc. of blood by multiplication by 0.0941.

Sample Calculation—2 cc. of blood were used in the analysis. 3 mg. of palladium chloride were placed in bulb E. The standard color prepared as directed previously was set at 20 mm. The unknown reading was 25 mm.

$$20 \times 2 = 25x$$
$$40 = 25x$$
$$x = 1.6 \text{ mg. palladium chloride remaining in bulb } E \text{ after reaction with carbon monoxide}$$

$3.0 - 1.6 = 1.4$ mg. palladium chloride reduced by carbon monoxide in 2 cc. of blood

$1.4 \times 50 \times 0.0941 = 6.59$ gm. hemoglobin per 100 cc. blood bound with carbon monoxide

Results

To test the accuracy of the method, bloods containing various concentrations of carbon monoxide hemoglobin were prepared and analyzed.   To prepare these bloods, it was first necessary to analyze normal dog blood for its content of hemoglobin and carbon monoxide hemoglobin.   By saturating a portion of the blood with carbon monoxide, and mixing this blood with the analyzed blood in different amounts, bloods of varying saturation of carbon monoxide were obtained.   The blood of laboratory dogs usually contained from 0.7 to 1.2 per cent of the hemoglobin bound with carbon monoxide.   This is interesting in view of similar results obtained with normal human blood.   After making allowance for the original carbon monoxide concentration of the blood, and the physically dissolved carbon monoxide contained in blood fully saturated with carbon monoxide, theoretical values for the content of carbon monoxide hemoglobin in these experimental bloods were obtained.   The calculated values of a series of bloods for carbon monoxide hemoglobin per 100 cc. were 0.57, 0.95, 1.29, 2.10, 2.43, 2.89, and 4.72.   The corresponding values for these bloods as determined by the present method were 0.56, 0.91, 1.23, 1.99, 2.21, 2.79, and 4.70.   Although certain improvements in the technique of the method have been made since these earlier analyses, there was on the whole a good agreement between the calculated values and those obtained by analyses.   In general, the analytical values were 3 to 4 per cent lower than the calculated values.

In Table I are reported the analyses of a series of bloods by the present method and the manometric method of Van Slyke and Robscheit-Robbins (8).   If one considers that an average blood contains between 15 and 16 gm. of hemoglobin, these analyses represent saturations of carbon monoxide ranging from 1 to 50 per cent.   These bloods were analyzed by the technique recommended in this paper, which we now believe to be the most reliable for all concentrations of carbon monoxide in blood.   This is mentioned at this point, since in response to requests for the details of the method, several laboratories have been furnished directions which differ somewhat from those recommended in this paper.   Many bloods were analyzed by the earlier technique in which sodium acetate or sodium chloride was used instead of

aluminum sulfate to flocculate the metallic palladium.   Many of these analyses agreed as well with the results obtained by the method of Van Slyke and Robscheit-Robbins as those recorded in Table I.   Occasionally, however, especially in the analysis of bloods containing high saturations of carbon monoxide, poor agreements were noted.   It was thought that the differences were due

TABLE I

Analysis of Blood for Carbon Monoxide

All values are expressed as gm. of hemoglobin combined with carbon monoxide per 100 cc. of blood.

Blood No.	Present method		Method of Van Slyke and Robscheit-Robbins
	Volume	Found	Found
	cc.	*gm.*	*gm.*
1	5	0.12	0.13
2	2	0.46	0.44
3	5	0.63	0 66
3	2	0.61	
4	5	0.82	0.83
4	2	0.82	
5	5	1.21	1.19
5	2	1.20	
6	5	1.98	1.98
6	2	2.01	
6	1	1.94	
7	5	3.37	3.30
7	2	3.30	
7	1	3.36	
8	5	4.92	5.02
8	2	4.86	
9	2	5.95	6.12
10	2	7.69	7.99
10	1	7.59	

to a poor separation of the metallic palladium from the palladium chloride.   This made an accurate colorimetric determination impossible.   With the use of aluminum sulfate, this difficulty was overcome.   The agreement between the present method and that of Van Slyke and Robscheit-Robbins is excellent.   It is also to be noted that practically identical results are obtained, regardless of the volume of blood employed in the analysis.

With two sets of the apparatus described in Fig. 1, five or six analyses can be made in duplicate in 1 day.   The cleaning of the apparatus between determinations is accomplished in a few minutes with soap and water.   The mercury used in the transference of the gas can be cleaned for reuse by washing with water.   If one is interested primarily in the qualitative detection of carbon monoxide in blood, one looks for particles of metallic palladium in the palladium chloride solution.   If these particles do not appear when 2 cc. samples of blood are used for analysis, the hemoglobin saturation with carbon monoxide is less than 3 per cent.   With 5 cc. samples of blood, saturations of carbon monoxide of 1 per cent or less will produce definite particles of metallic palladium in bulb E.   In order to detect the small amounts (0.05 to 0.1 mg.) of metallic palladium which would appear in the above cases, it is essential before each determination to wash the bulb E with a mixture of concentrated nitric and hydrochloric acids to remove every trace of metallic palladium remaining from the previous determination.   These particles which adhere to the inner surface of the bulb do not interfere with the quantitative determination.

Alternative Apparatus

A somewhat simpler but less convenient apparatus to replace bulb E with its 2-way stop-cock may be used.   A 1-hole stop-cock with a tube corresponding to F in Fig. 1 of about 2 to 3 mm. inside diameter and with a tube corresponding to tube I of capillary dimensions is needed.   Tube I is bent at right angles and is fitted by a 1-hole rubber stopper to a thick walled test-tube of approximately 30 cc. capacity.   The palladium chloride is measured directly into this tube, the stopper tightly fitted, and the tube evacuated.   This apparatus is then connected by the rubber tubing to tube D.   When the gas is transferred to the tube containing the palladium chloride, the blood-ferricyanide mixture must be brought to the capillary in the stop-cock but must not enter into the stop-cock.   With the stop-cock closed, the blood ferricyanide mixture may be washed from the tube F with a stream of water and thoroughly cleaned with a pipe cleaner.   Small portions of distilled water may then be introduced through tube F to force the gas into contact with the palladium chloride solution.

To obtain accurate results with this simpler apparatus great care must be taken to insure a quantitative transference of the palladium chloride to the filter paper in the removal of the metallic palladium.

Interfering Substances

It would seem that those methods for carbon monoxide, which depend upon slight differences in the shade of color produced when normal blood and blood containing carbon monoxide hemoglobin are treated with the same reagents, would fail if the blood had an abnormal color due to other causes.   Bloods obtained after cyanide poisoning, or after the inhalation of nitric oxide fumes, and bloods mixed with embalming fluids may be cited as examples. The presence of sodium cyanide in the blood does not interfere with the qualitative detection of carbon monoxide in blood.   The metallic palladium which is formed in the above method from a blood containing carbon monoxide is apparently the same whether 0, 100, 200, or 300 mg. of cyanide per 100 cc. of blood are present. However, when one attempts to proceed to a quantitative determination, the presence of 100 mg. of sodium cyanide per 100 cc. of blood gives values for carbon monoxide which are higher than the amount actually present.   This interference increases with increasing amounts of cyanide and appears to be due to the effect of the cyanide on the color reaction between potassium iodide and palladium chloride.

The presence of embalming fluid (containing formaldehyde, glycerol, and coloring material) in blood in the amount that one would expect to find in blood of embalmed bodies does not interfere with the quantitative determination of carbon monoxide by this procedure.

Palladium chloride solution through which nitric oxide gas is bubbled shows no indication of reduction.   Nitric oxide gas was passed through a blood which contained a small trace of carbon monoxide.   On reanalysis after this treatment no evidence of carbon monoxide could be obtained, since the carbon monoxide hemoglobin was oxidized to methemoglobin.   The important point, however, is that the presence of nitric oxide hemoglobin in blood analyzed by the present procedure produces no reduction of palladium chloride.

Since hydrogen sulfide converts palladium chloride into palladium sulfide, the effect of passing this gas through blood was studied. A stream of hydrogen sulfide was passed through a blood which had been analyzed for carbon monoxide. A reanalysis of the blood after this treatment gave results identical with the first analysis. Although it is unlikely that cases of poisoning with hydrogen sulfide would be confused with poisoning due to carbon monoxide, yet it is gratifying to know that sulfides present in the blood will not interfere with the determination.

SUMMARY

A simple and accurate method requiring inexpensive apparatus is described for the determination of carbon monoxide in blood. The method is based primarily on the fact that palladium chloride is reduced by carbon monoxide, and the excess of palladium chloride which is present is determined by a colorimetric procedure.

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THE FIRST DISSOCIATION CONSTANT, pK'₁, OF CARBONIC ACID IN HEMOGLOBIN SOLUTIONS AND ITS RELATION TO THE EXISTENCE OF A COMBINATION OF HEMOGLOBIN WITH CARBON DIOXIDE

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The question of the combination, other than as an acid, of some form of carbonic acid with hemoglobin has been reopened. The "combined" CO_2 in the blood is determined as the difference between the total CO_2 and the dissolved CO_2.

$$\text{Combined } CO_2 = \text{total } CO_2 - \text{dissolved } CO_2 \tag{1}$$

The total CO_2 is determined by adding acid to the solution and evacuating; the dissolved CO_2 is calculated from the solubility coefficient and the partial pressure of the CO_2 in equilibrium with the solution. The combined CO_2 so calculated has been generally assumed to be practically all in the form of HCO_3 . In 1928 Henriques advanced the hypothesis that a considerable amount of CO_2 may be bound with hemoglobin in a CO_2-Hb compound which he called carbhemoglobin. This hypothesis was entertained to explain the rapid taking up, or giving off, of CO_2 on the part of blood or hemoglobin solutions, which has since been shown to be of a catalytic nature (Van Slyke and Hawkins, 1930; Brinkman and Margaria, 1931; Brinkman, Margaria, Meldrum, and Roughton, 1932).

The equilibria in solutions containing hemoglobin, carbonic acid, and bicarbonate have been reexamined with this possibility in mind. In some of the experiments performed there is an apparent excess of the combined CO_2 as calculated in Equation 1 over the amount attributable to the bicarbonate, or carbonate, ions calculated by other methods. This apparent increase of combined CO_2 may be explained either (1) by a decrease of the activity coefficient

of the HCO_3^- in hemoglobin solutions or (2) by the presence of a certain amount of CO_2 combined with the hemoglobin, possibly in a compound of the type of Henriques' carbhemoglobin. On the latter assumption

$$(CO_2 \text{ combined}) = (HCO_3^-) + (CO_3^-) + (HbCO_2) \qquad (2)$$

The experimental methods employed by those who have studied this equilibrium include determinations of freezing point lowering, vapor tension, membrane equilibria, titration curves, and dissociation constants, with both the hydrogen and glass electrodes.

The osmotic coefficient of sodium bicarbonate or of sodium chloride in solutions containing hemoglobin has been studied by Stadie and Sunderman (1931) and by Stadie and O'Brien (1931) by determining freezing points, and by Margaria (1931, 1932) by Hill's vapor tension method at room temperature.

Both Margaria and Stadie and collaborators found that the presence of hemoglobin does not alter to an appreciable extent the osmotic coefficient. This led Margaria to the conclusion that the activity coefficient, which is thermodynamically related with the osmotic coefficient, also cannot be altered, and therefore that the apparent increase of the combined CO_2 has to be explained with the other alternative hypothesis; i.e., the combination of the hemoglobin with the CO_2 or with the HCO_3^- ions. These experiments would seem to indicate also that the amount of CO_2 combined with the hemoglobin must be so low as to be inappreciable in these solutions of relatively high bicarbonate content, since CO_2 so combined would be osmotically inactive.

When, however, a hemoglobin solution is equilibrated with two different mixtures of CO_2 first a lower one (0.4 per cent) and then a higher one (10 per cent), by Hill's vapor tension apparatus (Margaria, 1931), the increase in osmotic pressure is less than could have been predicted by the increase of combined CO_2 on the assumption that this was all in the form of bicarbonate. That is, whereas absolute values gave no indication either of any appreciable effect of the hemoglobin on the activity coefficient or of combination with the CO_2, differential values on solutions of low bicarbonate content indicate that as high as 40 per cent of the difference in the combined CO_2 may be bound to the hemoglobin.

In dialysis experiments with hemoglobin solutions, the distri-

bution of the combined CO_2 and of hydrogen ions on the two sides of the dialyzing membrane was such that the quantity of the CO_2 combined in the solution containing Hb was greater than could have been expected from its calculation from the Henderson-Hasselbalch formula on the assumption that all of the combined CO_2 was HCO_3^- (Adair, 1925, 1928; Henriques, 1928, 1931). Similarly the so called Donnan ratio for the distribution of ions between the inside and the outside of the blood cells, as calculated, is about 50 per cent greater for the bicarbonate ion than for the hydrogen ions (Van Slyke, Hastings, Murray, and Sendroy, 1925; see also Henderson, 1928). The Donnan ratio is also somewhat greater for chloride ions than for hydrogen ions and variations in both instances were originally considered as due to changes in activity coefficients of the ions concerned. The experimental relative distribution ratios were found to be as follows:

$$\frac{(a_H)_i}{(a_H)_c} = 0.77 \frac{(Cl)_c}{(Cl)_i} = 0.62 \frac{(CO_2 \text{ combined})_c}{(CO_2 \text{ combined})_i}$$

The titration curve of a sodium hemoglobinate solution in the pH range from 6 to 8 is apparently the same whether the titration has been with HCl or with CO_2 (Hastings, Sendroy, Murray, and Heidelberger, 1924; Van Slyke and Hawkins, 1930; Stadie and O'Brien, 1931). The HCl titration of reduced or carbon monoxide hemoglobin may be performed with the hydrogen electrode and the CO_2 titration by the hydrogen electrode or by calculating the pH values by means of the Henderson-Hasselbalch equation

$$pH = pK'_1 + \log (CO_2 \text{ combined}) - \log (CO_2 \text{ dissolved}) \tag{3}$$

The base bound by the hemoglobin is calculated as

$$(BHb) = (B_{total}) - (B_{combined\ CO_2}) \tag{4}$$

(B_{total}) represents a known amount of alkali added to a solution of isoelectric hemoglobin. Equation 4 represents the assumption that all the combined CO_2 was combined as $BHCO_3$ with part of the B_{total}, and that the rest of the B_{total} was combined with hemoglobin as BHb.

It would appear that, since titration curves with HCl and CO_2 are identical, with BHb calculated according to Equation 4 and

paH determined with the hydrogen electrode, all of the combined CO_2 is accounted for, and none is left over to combine with the hemoglobin as $HbCO_2$. This of course assumes that the combination of the hemoglobin with CO_2 is such that the CO_2 so combined is not free to combine with base. This is not necessarily true. For instance the linkage might be of the carbamate nature between bicarbonate ion and an NH_2 group inactive in the pH range studied.

$$R\text{—}NH_2 + HCO_3^- = R\text{—}HNCOO^- + H_2O \tag{5}$$

If the above happened to be the reaction, the base bound by the CO_2 in Equation 3 would be identical whether the HCO_3^- involved were attached to the hemoglobin or not. Thus the identical nature of the titration curve does not necessarily imply the impossibility of combination of hemoglobin with CO_2.

The determination of pK′₁ of Equation 3 in the presence of hemoglobin does, however, lead to results that must be explained either as variation in activity coefficients of the bicarbonate ions in the hemoglobin solutions or as a combination between CO_2 and hemoglobin. As far as we know these are the only two hypotheses which can satisfactorily account for the facts with which we are now acquainted. pK′₁ was demonstrated to be distinctly lower in a solution of laked horse red cells than in a salt solution of the same ionic strength by Van Slyke, Hastings, Murray, and Sendroy (1925). Stadie and Hawes (1928) made a careful and complete study of the effect of various types of hemoglobin, reduced or in combination with various gases, in various concentrations, upon the first dissociation constant of carbonic acid in solutions of varying ionic strength. They used a hydrogen electrode to determine the pH. The results showed a uniformly lower value for pK′₁ than in the absence of hemoglobin under conditions otherwise the same. These differences were ascribed to the influence of hemoglobin in diminishing the activity of the bicarbonate ions, and this influence was adequately described in terms of the Debye-Hückel theory of interionic forces in electrolyte solutions. The lower value of pK′₁ could also be explained if the combined CO_2 in Equations 1 and 2 included some CO_2 bound to the hemoglobin. The total combined CO_2 would thus exceed

the actual $BHCO_3$, and pK'_1, calculated on the assumption that all the combined CO_2 was $BHCO_3$, would be lower than the actual pK'_1. The value of pK'_1 in the presence of hemoglobin may, for convenience of expression, be termed pK'_1*.

If the hemoglobin combines with some form of carbonic acid, the quantity of $HbCO_2$ complex formed will be, according to the law of mass action, greater the greater the concentration of Hb, CO_2, or HCO_3^-, thus increasing the total amount of combined CO_2. It follows that for a constant Hb content the deviation of the value of the apparent pK'_1 in Equation 3 due to this combination must vary with variations of carbon dioxide and bicarbonate.

Such variation of pK'_1* was not shown by Stadie and Hawes' determinations because these authors worked largely within rather narrow limits of CO_2 tension and of base concentrations.

It seemed advisable to determine the apparent dissociation constant of carbonic acid in hemoglobin solutions of varying concentration in widely varying bicarbonate concentrations and CO_2 pressures. pH determinations on such solutions were made possible by the use of the glass electrode.

Preliminary experiments of this type were made recently by Margaria, Rowinski, and Goldberger (1933). They showed that the apparent increase of the (CO_2 combined) as calculated from the deviation of the determined pK'_1 of the carbonic acid from the theoretical value is greater when either the (CO_2 dissolved) or the (HCO_3^-) in solution is increased. Their results, however, as the authors themselves state, are not suitable for quantitative treatment.

Method—Crystalline horse hemoglobin was prepared by the method previously described (Ferry and Green, 1929; Green, 1931). Citrated horse cells were washed with hypertonic saline in a Sharples centrifuge and crystallized by the addition of HCl. After recrystallization the hemoglobin was thoroughly washed with water to remove most of the excess NaCl. Hemoglobin solutions of varying concentrations were prepared from these crystals, either by the addition of NaOH or by reduction in a vacuum. The concentration of hemoglobin was determined by the oxygen capacity or by nitrogen determinations by the Kjeldahl method, horse hemoglobin being assumed to contain 16.7 per cent nitrogen (Vickery and Leavenworth, 1928). The total base content was checked

by the manometric method of Van Slyke, Hiller, and Berthelsen (1927). Chloride determinations were carried out according to the Wilson and Ball (1928) modification of the Van Slyke technique.

Each of the hemoglobin solutions was equilibrated with various mixtures of different CO_2 content varying from 0.5 to 100 per cent. The equilibrations were carried out in tonometers for 20 minutes at 38°, according to the usual technique followed in this laboratory (Dill, in Henderson's monograph, 1928).

The pH of the solutions was measured with the glass electrode at 38°. Phosphate buffers were used as the standard, the pH values being calculated by means of the equation of Cohn (1927) with pK'_2 at 38° as 7.16. The total CO_2 was determined with the Van Slyke and Neill apparatus, and the CO_2 pressure by Haldane analyses. The dissolved CO_2 was calculated from the partial pressure and use of the solubility coefficient determined by Van Slyke *et al.* (1928), corrected for salt and hemoglobin concentrations. The amount of water present was calculated on the assumption that the specific volume of hemoglobin is 0.75. The solubility coefficient of CO_2 in H_2O is lowered 20 per cent for every mol of NaCl or $NaHCO_3$ present and the solubility calculated per gm. of H_2O is increased 10 per cent by 30 gm. of hemoglobin present per 100 gm. of H_2O and proportional amounts for lower quantities of protein. (CO_2 dissolved) $= 0.0320 \times pCO_2$ $(1 - 0.75$ Hb$)$ $(1 - 0.2$ [Na$^+$]$)$ $(1 + 0.33$ Hb$)$. (CO_2) is given in mM per liter, [Na$^+$] in mols per liter, pCO_2 in mm. of Hg, and Hb in gm. per cc.

The correction for hemoglobin was derived from experiments upon cell contents, but probably holds for hemoglobin solutions since other gases have also been found to be more soluble per gm. of water, in hemoglobin solutions than in pure water. The correction makes little difference in the calculation of the bound CO_2 at low CO_2 pressures but is significant at higher pressures.

First Dissociation Constant, pK′₁, of Carbonic Acid at 38°—pK'_1 for carbonic acid has been determined by Hastings and Sendroy (1925) and by Stadie and Hawes (1928) in solutions of varying total ionic strength, but with relatively constant $NaHCO_3$ concentrations (0.02 to 0.04 M). Since our experiments cover wider ranges of bicarbonate concentration and CO_2 pressure, it was neces-

sary to check their equation for variations of pK'_1 with electrolyte concentration under the conditions used in our experiments with hemoglobin. The procedure was exactly that observed in the experiments with hemoglobin, except that equilibration in the

TABLE I

Apparent First Dissociation Constant of Carbonic Acid at 38°

		CO₂			CO₃⁼	HCO₃⁻	pH	pK₁	
Na⁺	pCO₂	Dis-solved	Total	Com-bined				Deter-mined	Calcu-lated
M per l.	mm. Hg	mM per l.	mM per l	mM per l	mM per l.	mM per l			
0 030	23 8	0 768	30.4	29 6	0 2	29.4	7 801	6.218	
	63 8	2 03	31 6	29 6	0 1	29 5	7 409	6 248	
	193 5	6.16	35 6	29 4		29 4	6 928	6 249	
								6.239	**6.243**
0 050	13 2	0.419	49 2	48 8	0 9	47 9	8 265	6 208	
	53 3	1 69	51 5	49 8	0 3	49 5	7 701	6 234	
	212	6 73	57.1	50 4	0 1	50 3	7 101	6 228	
	806	25 6	76 1	50 5		50 5	6 525	6 230	
								6.225	**6.218**
0 082	17.21	0.544	79 2	78 7	2 0	76.7	8 309	6 160	
	20 8	0 656	79 6	78 9	1 7	77.2	8.240	6 171	
	61 7	1.95	83 2	81 2	0 6	80 6	7 786	6.170	
	186 4	5 88	87.0	81 1	0 3	80 8	7 319	6 181	
								6.171	**6.187**
0 128	13 8	0 429	123.6	123 1	6.7	116.4	8 575	6 142	
	18 5	0 576	125 0	124 6	5 1	119 5	8 463	6 157	
	60 4	1.88	129.0	127 1	1 9	125 2	8 001	6.178	
	185 5	5 78	133.5	127 7	0 6	127.1	7 523	6.181	
								6.165	**6.150**
0 130*	21 4	0 666	30 8	30.1	0 3	29 8	7.803	6.153	
	60 8	1.89	31 4	29 5	0 1	29 4	7 360	6 169	
	189.3	5.89	36 0	30 1		30.1	6 868	6 159	
								6.160	**6.150**

The figures in bold-faced type represent averages.

* Cl⁻ = 0.100.

water bath was carried out for a longer period, at least 45 minutes. The results are given in Table I. The "Calculated" values are those given by the limiting Debye-Hückel equation as used by Hastings and Sendroy

$$pK'_1 = 6.33 - 0.5 \sqrt{\mu} \qquad (6)$$

TABLE II

pK'₁ of Carbonic Acid in Reduced Hemoglobin Solutions at 38°

pCO₂	CO₂			pH	pK'₁*	HCO₃⁻	CO₃⁼	HbCO₂
	Dissolved	Total	Combined					

Experiment 1. Hb 3.17 mM per l.; Na⁺ 0.0028 M; Cl⁻ 0.0016 M; CO₂ factor 0.0312; pK'₁ 6.30–6.29

mm. Hg	*mM per l.*	*mM per l.*	*mM per l.*			*mM per l*	*mM per l.*	*mM per l*
9.20	0.287	1.33	1.04	6.565	6.005	0.53		0.51
29.9	0.934	3 17	2 24	6 485	6 103	1 43		0 81
99.1	3 09	6.80	3.71	6 247	6 168	2.73		0 97
310	9.67	15.25	5 58	5 944	6.183	4 36		1 22
814	25 4	32 4	7.0	5 672	6.233	6.13		0 87

Experiment 2. Hb 3.2 mM per l.; Na⁺ 0.024 M; Cl⁻ 0.0016 M; CO₂ factor 0 0311; pK'₁ 6 25

10 2	0.318	13.58	13 26	7 817	6 197	11 67		1.59
28.4	0.884	16 95	16 07	7.465	6.205	14 50		1.57
97.6	3.04	22.5	19 46	7 010	6 204	17.48		1.98
302	9.40	31.3	21 9	6.601	6.234	21.1		0.8
809	25.1	49.6	24 5	6 225	6.235	23.7		0.8

Experiment 3. Hb 2.92 mM per l.; Na⁺ 0 114 M; Cl⁻ 0 0017 M, CO₂ factor 0.0306; pK'₁ 6 16, pK'₂ 9.85

9.9	0 303	84.1	83 8	8 583	6.132	80.3	4.3	−0 8
28 1	0 861	90 4	89.5	8 163	6.146	86.9	1 8	0 8
99.0	3 03	96.8	93 8	7.653	6.163	90.0	0.6	2 2
303.5	9.29	106.7	97.4	7.205	6 185	102 9	0.2	−5.7
796	24.4	125	100.6	6 792	6.177	104.2		−4.2

Experiment 4. Hb 8.39 mM per l.; Na⁺ 0.005 M; Cl⁻ 0.0032 M; CO₂ factor 0.0300; pK'₁ 6.29–6 27

10.13	0.304	1.72	1.42	6.680	6.011	0.75		0.67
33.0	0.99	4.39	3 40	6 582	6.027	1 89		1 51
98 6	2.96	9.71	6 75	6.442	6.084	4.21		2.54
294.7	8.85	20.4	11.55	6 238	6.123	8.05		3.50
815	24 5	40 3	15 8	5 963	6.152	12 0		3.8

Experiment 5. Hb 8.75 mM per l.; Na⁺ 0.025 M; Cl⁻ 0.0032 M, CO₂ factor 0.0300; pK'₁ 6.25

11.0	0.33	7.36	7.03	7.445	6.117	5.17		1.86
30.9	0.927	12.06	11 13	7 236	6.157	8.97		2.16
104 0	3 12	20.85	17.73	6.907	6.152	14.17		3.56
802	24.06	53.2	29.14	6.253	6.167	24.24		4.90

TABLE II—*Concluded*

pCO₂	CO₂			pH	pK'₁*	HCO₃⁻	CO₃⁻	HbCO₂
	Dissolved	Total	Combined					

Experiment 6. Hb 8.0 mM per l.; Na⁺ 0 120 M; Cl⁻ 0.032 M; CO_2 factor 0.0293; pK'₁ 6.16; pK'₂ 9.84

mm. Hg	mM per l.	mM per l.	mM per l			mM per l.	mM per l	mM per l.
15.38	0.45	68 6	68 1	8.299	6.123	61 9	1.7	4 5
32.8	0.96	72.9	71 9	8 015	6.141	68.6	1 0	2 3
101.3	2.97	83.7	80.7	7 585	6.150	79 0	0.4	1 3
297.0	8.70	99.3	90 6	7.172	6 155	89 4	0 2	1.0
802 6	23.5	123	99 5	6.780	6 153	98 0	0 1	1.4

Experiment 7. Hb 16 0 mM per l ; Na⁺ 0.032 M; Cl⁻ 0 0053 M, CO_2 factor 0.0277; pK'₁ 6.24

8 74	0.242	3.55	3 31	7 177	6.041	2.09		1 22
27.8	0.77	8.57	7 80	7 068	6 062	5 19		2 61
94 85	2.63	18 18	15 55	6 854	6.082	10 8		4 75
300.5	8.33	35.1	26 8	6 595	6 087	18.9		7.9
825	22.9	59 5	36 6	6 323	6 121	27.7		8.9

Experiment 8. Hb 15.3 mM per l , Na⁺ 0 119 M; Cl⁻ 0 0004 M, CO_2 factor 0.0272, pK'₁ 6 16, pK'₂ 9.84

19 4	0.538	49 3	48.8	8.048	6 090	41 6	0 6	6.6
38 1	1 04	56 6	55 6	7.846	6 118	50 4	0 5	4 7
104.0	2 83	73.4	70 6	7 508	6 120	63.2	0 3	7.1
337 5	9 18	96 7	85 5	7 118	6 149	83 2	0 1	2 3
806 5	21.95	123.9	101 9	6 797	6.131	95.0	0 1	6.8

μ is the ionic strength calculated per liter, and 6.33 the pK₁ at 0 concentration of electrolyte. The average results at each ionic strength have been plotted along the straight line in Fig. 2 which represents Equation 6.

The pH was so high in some of the solutions that it was necessary to correct for the concentration of carbonate ions present. This was done, assuming Hastings and Sendroys' (1925) equation,

$$\text{pK}'_3 = 10.22 - 1.1\sqrt{\mu} \tag{7}$$

to hold under these conditions, and the CO_3^- concentration was calculated from Equation 10.

The curve thus seems to be the same whether the ionic strength is increased (up to 0.13 M) by the addition of NaCl or by NaHCO₃.

There is a drift in pK′$_1$ values in only the most concentrated bicarbonate solution. This drift is in such a direction as to increase the possible CO_2 bound by hemoglobin as calculated below, but since it would take many more determinations to establish its magnitude, it has been disregarded in the calculations.

Variation of pK′$_1$ for Carbonic Acid in Solutions of Varying Reduced Hemoglobin Concentration and Carbon Dioxide Tension and Base Content—The variation of pK′$_1$* with CO_2 tension and bicarbonate concentration in reduced hemoglobin solutions is shown by the results given in Table II. The solutions contained
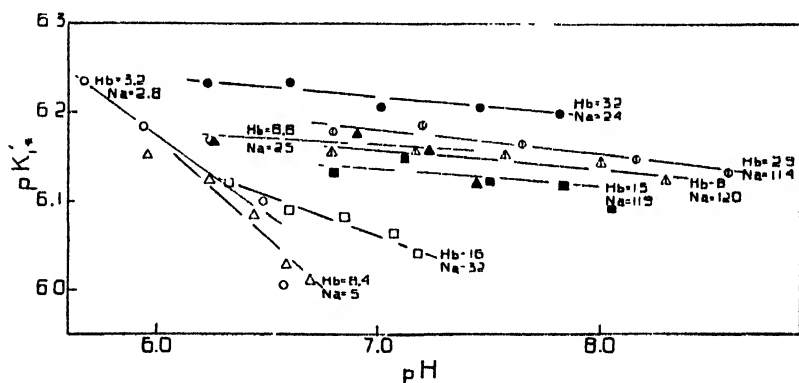
Fig. 1. pK′$_1$ of H_2CO_3 in reduced hemoglobin solutions of varying pH. The concentrations of Na and Hb are given as mM per liter.

reduced hemoglobin in concentrations up to 25 per cent, sodium bicarbonate up to 0.1 M, and free carbon dioxide up to 1 atmosphere.

In the data given in Fig. 1, pK′$_1$* has been plotted against pH. The variation for a given solution is apparently linear. The slope of the line is greater the lower the hemoglobin concentration and the lower the ionic strength   Increase in CO_2 tension increases the pK′$_1$*, regardless of whether the pH is greater or less than the isoelectric point of the hemoglobin.

The difference between the pK′$_1$ values of the carbonic acid in the presence and in the absence of hemoglobin *at constant ionic strength* may be called ΔpK.

$$pK'_1 - pK'_1* = \Delta pK \tag{8}$$

ΔpK increases with the hemoglobin concentration at the same bicarbonate concentration. This was also found by Stadie and Hawes.   In these experiments ΔpK varies inversely with the bicarbonate concentration, becoming negligible at 0.1 M NaHCO₃.

Variations of ΔpK may be explained either by eventual changes in ionization of the hemoglobin or by the combination of the hemoglobin with some form of CO₂.   The effect of the ionization of the hemoglobin, if of any importance, is presumably constant near the isoelectric point.   To exclude any variations of $pK_1'^*$ due to this effect, the data in Fig. 1 have been interpolated or extrapolated to
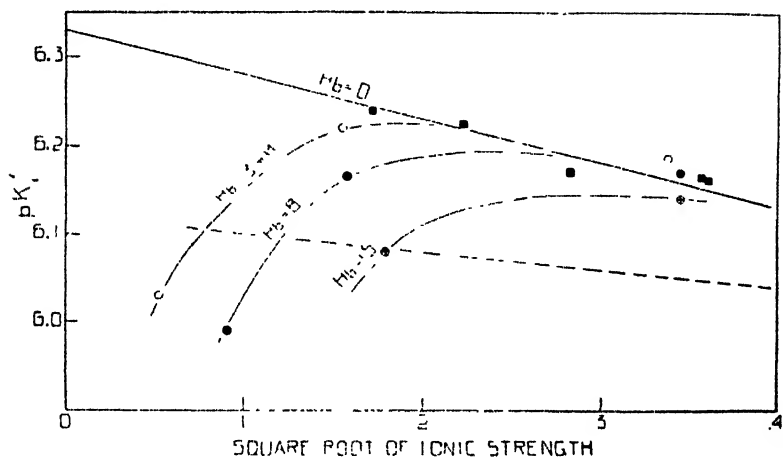
FIG. 2. pK_1' of H_2CO_3 in reduced hemoglobin solutions at constant pH and varying ionic strength.

pH 6.8 and $pK_1'^*$ plotted at constant hemoglobin concentration against the square root of the ionic strength in Fig. 2.   The upper straight line in Fig. 2 is drawn according to Equation 7 and represents the variation of pK_1' for H_2CO_3 in solutions of the same ionic strength but in the absence of hemoglobin, as reported in the preceding section of this paper.

The variation of $pK_1'^*$ with ionic strength is in the opposite direction from that found by Stadie and Hawes (1928) for bicarbonate concentrations low in relation to the concentration of the hemoglobin; that is, $pK_1'^*$ increases with increasing ionic strength at low ionic strengths.   The dash line in Fig. 2 is their curve for

the variation of pK'$_1$* with ionic strength in the presence of 13 mM of reduced hemoglobin (Stadie and Hawes, 1928, Table VI, p. 282) recalculated with the solubility coefficients used in this paper.   The essential difference between our experiments and those of Stadie and Hawes lies in the salt used for increasing the ionic strength. Variation in ionic strength in our experiments is due largely to changes in NaHCO$_3$; in their experiments, largely to NaCl concentration.

TABLE III

pK'$_1$ of Carbonic Acid in Reduced Hemoglobin Solutions of Constant Ionic Strength but Varying Bicarbonate Concentration*

pCO$_2$	CO$_2$			pH	pK'$_1$*	HCO$_3^-$	CO$_3^=$	HbCO$_2$
	Dissolved	Total	Combined					

Experiment 9. Hb 12 6 mM per l.; Na$^+$ 0 104 M; Cl$^-$ 0 080 M; CO$_2$ factor 0 0284, pK'$_1$ 6.17

mm. Hg	mM per l.	mM per l	mM per l			mM per l	mM per l	mM per l.
20 94	0.595	6 73	6 14	7.085	6.069	4.89		1.25
63.0	1.79	14.25	12 46	6.934	6 093	10 40		2.06
158.4	4.50	24 6	20.1	6 759	6.110	17.5		2.6
350	9 93	37 8	27.9	6 594	6 145	26 4		1.5

Experiment 10. Hb 12.5 mM per l.; Na$^+$ 0 114 M; Cl$^-$ 0 003 M; CO$_2$ factor 0 0284; pK'$_1$ 6 16, pK'$_2$ 9.87

42.8	1 217	61.0	59.8	7 871	6 179	62.5	0.6	−3 3
131.9	3 74	78 7	75.0	7.481	6 180	78 3	0.3	−3 6
281	7.97	94 1	86 1	7 210	6 177	89.5	0 2	−3.6
801	22.8	122.5	99.7	6.827	6.187	106	0 1	−6 4

To show that these differences at high ionic strength, apparently due to the nature of the salt, are not caused by differences in technique in the two investigations, we have determined pK'$_1$* at a total ionic strength of 0.1, due in one experiment to the presence of NaHCO$_3$ and in the other to 0.08 M NaCl and 0.02 M NaHCO$_3$. The results are given in Table III.   In the solutions containing only bicarbonate, the pK'$_1$* is close to the value in the absence of hemoglobin at the same ionic strength, while in the solution with only 0.02 M NaHCO$_3$ pK'$_1$* is lower than in the absence of hemoglobin and varies with the CO$_2$ tension.   The difference between the two pK'$_1$* values at constant ionic strength but varying

amounts of bicarbonate is therefore real and not due to technical differences.   The magnitude of the differences also is, at the same pH, approximately the same as that shown in Fig. 2.

The dissociation constant of carbonic acid in reduced hemoglobin solutions exhibits, then, two peculiarities other than a variation with hemoglobin concentration or ionic strength.   These are a variation with CO_2 tension and a variation with relative bicarbonate concentration.   Although the activity coefficient of HCO_3^- might be expected to vary with bicarbonate concentration, even at constant ionic strength, this is not the case in dealing with mixtures of NaCl and $NaHCO_3$.   This does not exclude such a possible variation in sodium bicarbonate and sodium hemoglobinate mixtures.   In such a case the picture of interionic forces involved necessary to explain the new facts has to be much more complicated than that already presented in the previous literature. On the other hand, the variation of $pK'_1{}^*$, greater in lower bicarbonate concentrations and dependent upon the CO_2 tension or pH at constant ionic strength and hemoglobin concentration, could be simply explained on the hypothesis of the presence of a certain amount of CO_2 bound with the hemoglobin; if we assume that the relative amount of CO_2 bound in the $HbCO_2$ complex over the total combined CO_2 is greater for lower bicarbonate concentrations, the deviation brought by this phenomenon on the ratio (CO_2 combined) / (CO_2 dissolved) and consequently on the calculated value of $pK'_1{}^*$ (Equation 3) will, in fact, be greater for low bicarbonate concentrations.

Quantitative Estimation of CO_2 Bound by Reduced Hemoglobin—It is probable that hemoglobin does affect the dissociation of carbonic acid, but let us assume that this effect is negligible and calculate the amount of HCO_3^- that would have to be combined with the hemoglobin in order to obtain the variation in $pK'_1{}^*$ found. This assumption of a negligible electrostatic effect is supported by the absence of an appreciable effect of hemoglobin on the osmotic coefficient of HCO_3^- and of NaCl.

The $[HbCO_2]$ is calculated by Equation 2, $[HCO_3^-]$ and $[CO_3^-]$ being subtracted from the total combined CO_2. $[HCO_3^-]$ is calculated by a rearrangement of the Henderson-Hasselbalch equation

$$[HCO_3^-] = [CO_{2\text{dissolved}}] \times 10^{pH - pK'_1} \tag{9}$$

where pK′₁ has the values, given by Equation 6, which it has in solutions of the same ionic strength but without hemoglobin. $[CO_3^-]$ is calculated by the equation

$$[CO_3^-] = [HCO_3^-] \times 10^{pH - pK'_2} \tag{10}$$

where pK′₂ has similarly the values given by Equation 7. The quantity of CO_3^- is significant only at pH values above 7.5 and in the more concentrated bicarbonate solutions. The values for $(HbCO_2)$ so calculated are given in the last column of Tables II and III.

In solutions of low ionic strength, the CO_2 bound to the hemoglobin increases with the bicarbonate concentration. At high bicarbonate concentrations the $(HbCO_2)$ fluctuates and even becomes negative in the solution containing the lowest amount of hemoglobin. These fluctuations are due to the estimation of small absolute values by differences of relatively large quantities; for example, a variation of pH or of pK′₁ of 0.03 in Experiment 3 (Table II) would change the negative values to positive with 1 mol of CO_2 bound to each mol of Hb. The CO_2 solubility factor also becomes highly important; calculations which ignore the increased solubility in water, due to the presence of hemoglobin, increase $(HbCO_2)$ about 100 per cent in these solutions high in bicarbonate. Also, it is not at all impossible that the presence of hemoglobin may alter the activity coefficient of the HCO_3 in such a way as to increase rather than decrease pK′₁*. In any case, although most of the equilibria in high bicarbonate concentrations, especially in the presence of higher quantities of hemoglobin, may be interpreted on the basis of the formation of $HbCO_2$, they are not suitable for quantitative analysis.

In Fig. 3 the values for CO_2 combined with hemoglobin are plotted against the (HCO_3^-) concentration for the three concentrations of reduced hemoglobin employed at the lower bicarbonate concentration; i.e., Experiments 1, 4, 5, and 7. In order to put all the values on one figure the $HbCO_2$ has been plotted as per cent of total Hb. We have also plotted Stadie and Hawes' data, given in their Tables V and VI, on the same curve, using only the points to which no NaCl had been added, calculating $HbCO_2$ as above with the same constants. The curves drawn

through the points for each concentration of hemoglobin are rectangular hyperbolas with horizontal asymptotes at a value of $HbCO_2$ equal in mols to the mols of hemoglobin present, on the assumption of a molecular weight of 16,800. Thus, 1 molecule of CO_2 combines with the same amount of hemoglobin as does 1 molecule of O_2; the CO_2 "capacity" equals the O_2 capacity.
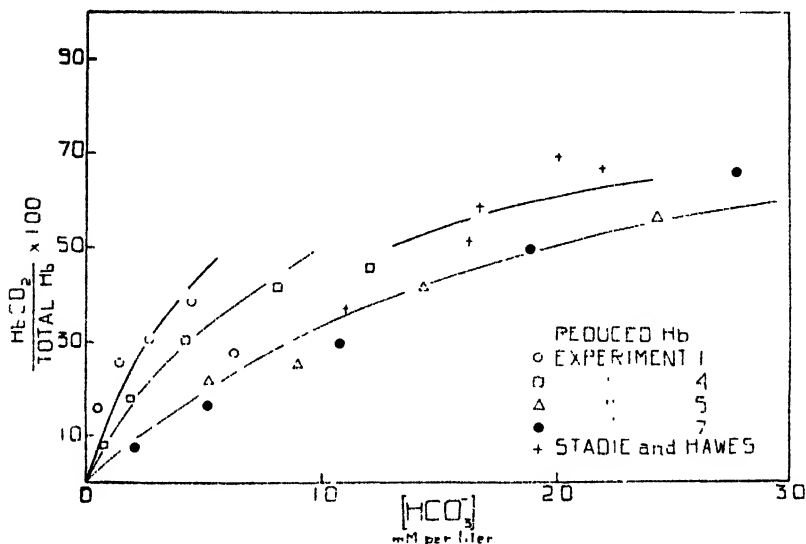
FIG. 3. $HbCO_2$ in reduced hemoglobin solutions

The curves may be represented by the relation

$$\frac{(Hb)\,(HCO_3^-)}{(HbCO_2)} = k \tag{11}$$

where Hb = total Hb − $HbCO_2$. The constant k varies with the hemoglobin concentration and the ionic strength. This relation may be incorrect since it is based only on the more accurate data in low bicarbonate concentrations; but, if valid, it represents the equilibrium

$$Hb + HCO_3^- = HbCO_2 \tag{12}$$
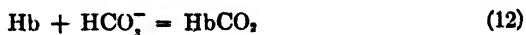
Equation 12, in order to show the taking up of a negative charge by the combination product, might more properly be written

$$Hb + HCO_3^- = HbHCO_3^- \tag{13}$$

or

$$Hb + CO_2 + OH^- = CO_2HbOH^- \qquad (14)$$
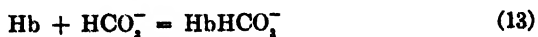
but since we hold no final brief for the mechanism of combination, for the sake of simplicity in this paper we shall call $HbCO_2$ the product of the binding, independent of the form of CO_2 combined.

Estimation of CO_2 Bound by Oxyhemoglobin—As Stadie and Hawes (1928) and Henriques (1928) showed that the discrepancy between pK$'_1$* and pK$'_1$ is greater for reduced hemoglobin solutions
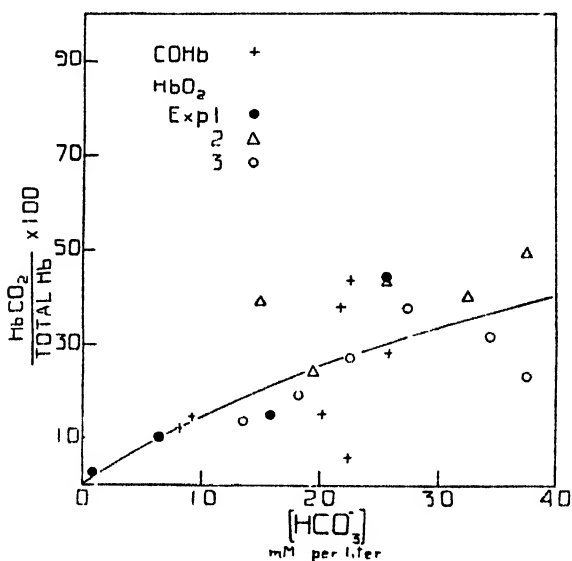
FIG. 4. HbCO$_2$ in oxyhemoglobin solutions

than for carboxyhemoglobin, it seemed desirable to see whether the behavior in oxyhemoglobin solutions showed the same mechanism and whether the capacity of oxyhemoglobin for binding CO_2 differed from that of reduced hemoglobin.

Determinations carried out on oxyhemoglobin solutions are reported in Table IV. Oxyhemoglobin also decreases the value of pK$'_1$ for carbonic acid and the results may again be interpreted as the combination of CO_2 with hemoglobin. In Fig. 4 the amount of CO_2 bound with the hemoglobin, calculated as in the presence of reduced Hb, is plotted against the bicarbonate concentration

as for Fig. 3.   This function may also be represented as for the reduced hemoglobin solutions by a rectangular hyperbola; only

TABLE IV

First Dissociation Constant of Carbonic Acid in Oxyhemoglobin Solutions

pCO₂	CO₂			pH	pK₁′*	HCO₃⁻	HbCO₂
	Dissolved	Total	Combined				

Experiment 1   Hb 10 mM per l , Na⁺ 0 041 N, Cl⁻ 0 005 N; CO₂ factor 0 0292; pK₁′ 6.23

mm. Hg	mM per l	mM per l	mM per l			mM per l.	mM per l.
3 01	0.088	2 18	2.09	7 57	6 19	1 93	0 16
16 76	0 49	7 96	7 47	7 35	6 17	6 47	1 0
81 5	2 41	19 8	17 4	7 05	6 19	15 9	1 5
340 5	9 95	41 0	30 0	6 64	6 16	25 6	4 4

Experiment 2   Hb 10 52 mM per l , Na⁺ 0 055 N, Cl⁻ 0 005 N; CO₂ factor 0 0294, pK₁′ 6 21

12 54	0 369	19 6	19 2	7 822	6 106	15 1	4 1
24 7	0 726	22 6	21 9	7.638	6 169	19 4	2 5
69 9	2 05	32 5	30 4	7 311	6 141	25 8	4 6
153 5	4 51	41 2	36 7	7 068	6 157	32 5	4 2
310 8	9 15	52 2	43 0	6 827	6 155	37 8	5 2

Experiment 3   Hb 9 29 mM per l , Na⁺ 0.053 N, Cl 0 009 N, CO₂ factor 0 0293; pK₁′ 6 22

12 05	0 354	14 87	14 52	7 794	6 180	13 25	1 27
29 9	0 876	20 95	20 07	7 539	6 179	18 3	1 77
55 0	1 61	26 6	25 0	7 366	6 177	22 5	2 5
111 0	3 25	34 1	30 9	7 147	6 167	27 4	3 5
230	6 75	44 4	37 6	6 930	6.184	34 7	2 9
334	9 80	49 8	40 0	6 807	6 196	37 8	2 2

Experiment 4   Hb 9 0 mM per l ; Na⁺ 0 073 N, Cl⁻ 0 009 N, CO₂ factor 0 0293; pK₁′ 6 195

17 9	0 525	37 4	36 9	8 033	6 186	36.2	0 7
31 1	0 911	40 7	39 8	7.827	6 187	39 0	0 8
59 0	1 70	47 2	45 5	7 603	6.176	43.5	2 0
114.6	3.36	54 0	50 64	7 356	6 179	48 7	1 9
371	10 89	75.0	64.1	6 945	6 175	61 2	2.9

the value of the constant k is different, corresponding to a greater flatness of the curve; the asymptotic value for HbCO₂, however, is the same as for the reduced hemoglobin.

In Fig. 4 are also plotted certain of the data of Stadie and Hawes on HbCO at approximately the same hemoglobin concentration. The values in their Tables II and III for which no NaCl has been added have been recalculated with the same constants as used in this paper.

Effect of Bicarbonate on the Oxygen Dissociation Curve—If the introduction of the oxygen into the hemoglobin molecule affects the binding of the CO_2 with hemoglobin, the reverse phenomenon
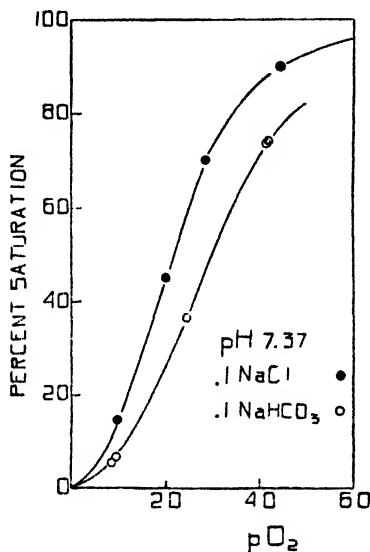
FIG. 5. Oxygen dissociation curves of hemoglobin in NaCl and NaHCO$_3$ at the same pH and ionic strength (0.1 M).

is to be expected; *i.e.*, the presence of the CO_2 on the molecule of the hemoglobin affects the oxygen binding of the hemoglobin.

An experiment was carried out for this purpose with two solutions in which hemoglobin concentration, ionic strength, and pH were approximately constant. The oxygen dissociation curve of a solution containing NaCl but free from CO_2 and HCO_3^- was found to differ very sensibly from the dissociation curve of a second solution in which the hemoglobin was presumably all in the $HbCO_2$ form, this being obtained by means of a high content of bicarbonate in solution and of a proportionate amount of CO_2. In

Fig. 5 are plotted the two oxygen dissociation curves, in which the one free from CO_2 has been corrected for the small deviation in pH, from the data given by Green and Talbott (1933) on similar hemoglobin preparations.

The difference between the two curves is very large: 50 per cent concentration is reached for the CO_2-free hemoglobin solution at a pressure of oxygen 8 mm. lower than for the $HbCO_2$ solution. This variation would seem rather large for a specific ion effect, although it is, of course, not impossible. However, it is not to be expected if the titration curves of NaHb with HCl and CO_2 are identical in the physiological range. The difference in the two curves is in the direction expected if CO_2 does combine with Hb.

The deviation due to the presence of CO_2 is also in the same direction as the one given by an increase in hydrogen ion concentration. The variations in the oxygen dissociation curves of blood or hemoglobin solutions due to changes in the CO_2 content have been until now attributed to the changes in pH. Evidently this does not account for the whole change in such conditions, if there is a $HbCO_2$ compound and the combination with CO_2 increases the difficulty of combination with O_2.

Mechanism of the Binding of CO_2 with Hb—Henriques (1928, 1929) originally indicated a mechanism of the carbamino type for the binding of the CO_2 with hemoglobin, such as the one studied by Faurholt (1925) for the binding of the CO_2 with amino acids or other amino bases; however, he had no evidence to support this hypothesis. The mechanism of the carbamino binding may be expressed, after Faurholt, by the following reactions

(a) $^+Am^- + HCO_3^- = amate^-$

(b) $CO_2 + 2\ Am^- = AmH^+ + amate^-$
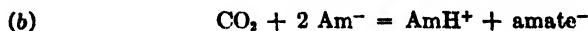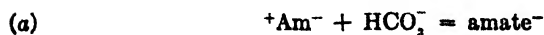
where $^+Am^-$ is the isoelectric amine, amate$^-$ is the carbamate radical dissociated as acid, and AmH^+ the amino acid radical dissociated as base. This binding is due to the $-NH_2$ group, in the following way, for reaction (a):

(c) $-NH_3^+ + HCO_3^- = -NHCOOH + H_2O$

or

              $-NH_2 + HCO_3^- = -NHCOO^- + H_2O$
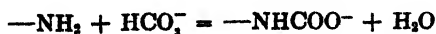
In the hemoglobin molecule there are many free NH_2 groups, while it appears that the amount of CO_2 which can be bound is 1 mol of CO_2 per 1 mol of Hb.   It does not seem likely that only one of the NH_2 groups of the hemoglobin molecule has the property of binding CO_2, unless, of course, it is peculiarly arranged in the molecule.

The effect of the CO_2 on the binding of the oxygen with the hemoglobin may suggest that this binding occurs in a point of the Hb molecule in immediate proximity to the point where the O_2 binds, possibly on the prosthetic group.   A series of determinations

TABLE V

$pK′_{1_{H_2CO_3}}$ in 1 Per Cent Hematin Solution in 0.04 N NaOH

pCO_2	CO_2			pH	$pK_1′$	
	Dissolved	Total	Combined		Determined	Calculated
mm. Hg	mM per l.	mM per l.	mM per l.			
13.4	0 423	8 69	8 27	7.547	6 256	
29 3	0 924	11 82	10 90	7 333	6 261	
58.2	1 83	11 89	10 06	6 980	6 240	
64 0	2.02	15 13	13 11	7 065	6 252	
103 8	3 29	16.90	13 61	6.850	6 233	
203 8	6 41	20.2	13.81	6.575	6 243	
207	6.53	23 5	17 0	6 565	(6 150)*	
Average....................			.. ..		6 25	6 23

* Not included in the average.

similar to those performed on Hb solutions has been carried out on a 1 per cent hematin solution, which is equivalent to a 25 per cent hemoglobin solution.   The hematin was dissolved in 0.04 N NaOH.   The experimental data are reproduced in Table V. $pK′_1$ varies from 6.23 to 6.26.   This would seem to indicate no appreciable effect either of electrostatic forces or of combination.   Thus the group combining with bicarbonate would seem to be near the attachment of the hematin to the globin (since oxygenation is affected) but not on the hematin itself.

This would apparently contradict Henriques' (1933) latest theory of combination as of a complex Hb-ferro salt.

Under the circumstances the chemical nature of the binding of CO_2 or HCO_3^- with hemoglobin would still seem to be unknown.

Rôle of HbCO₂ in the Blood — The physiological significance of the combination of CO_2 with hemoglobin may be best indicated by an example as given in Table VI. The values for combined CO_2 are as originally calculated. The pH, however, has been recalculated with pK'_1 for serum 6.10 (Hastings, Sendroy, and Van Slyke, 1928). pK'_1 in cells has been assumed to be 5.98 for arterial and 5.95 for venous blood. These values are somewhat arbitrary but it is certain that the value for oxygenated blood should be higher than for reduced. Stadie and Hawes (1928) found the difference between the values for reduced cells and those

TABLE VI

Calculation of Relative Concentration of HbCO₂ in Human Blood at Rest from Data of Henderson (1928)*

	Arterial		Venous		Δ = venous − arterial		
	Serum	Cells	Serum	Cells	Serum	Cells	Whole blood
pH	7.421	7 205	7 399	7.157			
CO_2 combined, mM per l...	15 29	6 03	16 07	6 65	0 78	0.62	1 4
HCO_3^-, mM per l	15 29	4 59	16 07	4.68	0.78	0 09	0 87
$HbCO_2$, mM per l . . .		1 44		1.97		0 47	0.47
$HbCO_2/CO_2$ combined. .		0 25		0 30		0 76	0.33

* Table 22, p. 201, blood of C. V. C.

saturated with CO to be about 0.1 for various bloods, and the value for reduced cells 5.87, whereas Van Slyke, Hastings, Murray, and Sendroy (1925) obtained an average value of 5.93 in reduced horse cells. This value should vary with the bicarbonate concentration and is dependent upon the value chosen for α_{CO_2}.

If the true pK'_1 in cells be assumed to be 6.10, the HCO_3^- and $HbCO_2$ can be calculated as in Tables I to IV. All values are given in mM per liter of blood.

From Table VI it appears that one-quarter to one-third of the combined CO_2 in the red cell is in the form of $HbCO_2$, although only 5 or 10 per cent of the total in the blood· is in that form. Three-quarters of the change in combined CO_2 in the cells and one-third of the total change in the blood between arterial and

venous blood may be regarded as due to the combination of CO^2 with hemoglobin. Thus, in reduction hemoglobin not only becomes a weaker acid setting free base to combine with CO_2, but it increases its own ability to combine with HCO_3^- or CO_2.

SUMMARY

1. The apparent first dissociation constant $pK'_1{}^*$ of carbonic acid has been determined in the presence of widely varying concentrations of hemoglobin, of bicarbonate, and of CO_2, the pH of the mixtures being determined with the glass electrode.

2. At constant ionic strength and hemoglobin concentration $pK'_1{}^*$ varies approximately linearly with the pH.

3. The difference between $pK'_1{}^*$ in the presence of, and pK'_1 in the absence of, hemoglobin at constant ionic strength is greater the higher the hemoglobin concentration.

4. At very low ionic strengths $pK'_1{}^*$ at constant pH increases with increasing ionic strength. This is a variation in the opposite direction from that previously described as following from the Debye-Hückel theory of interionic forces.

5. At high ionic strengths $pK'_1{}^*$ is lower the lower the proportion of $NaHCO_3$ making up the total electrolyte concentration.

6. If the deviation of $pK'_1{}^*$ from the value in the absence of hemoglobin, pK'_1, be assumed to be entirely due to the combination of CO_2 with hemoglobin and this quantity be calculated, the amount bound for low ionic strength increases with increase in the calculated bicarbonate concentration.

7. The difference between pK'_1 and $pK'_1{}^*$ is greater in reduced than in oxyhemoglobin solutions. This may be interpreted to mean that the combination of CO^2 with hemoglobin takes place more easily in reduced than in oxygenated solutions.

8. Reciprocally, the combination of O_2 with hemoglobin is affected by the combination of the CO_2. Oxygen dissociation curves at constant pH and ionic strength in $NaHCO_3$-CO_2 solutions are definitely moved to the right of curves in the absence of CO_2 under identical conditions of ionic strength, pH, and hemoglobin concentration.

9. pK'_1 in a 1 per cent hematin solution of 0.04 N Na^+ is the same as in the absence of hematin in a solution of the same ionic strength.

10. The changes in combined CO_2 in oxygenation of the blood

in physiological circumstances may be due not only to changes in pH, but to changes in the ratio $HbCO_2/Hb$, since reduced hemoglobin combines with more HCO_3^- than oxyhemoglobin under the same conditions.

11. The real existence of a binding of the CO_2 with hemoglobin is suggested by the variation of $pK_1'^*$ with pH, by the increase of $pK_1'^*$ with ionic strength at low ionic strengths, and the effect of HCO_3^- on the oxygen dissociation curves. These results, if explained on the theory of interionic forces, require a much more complicated theory than any heretofore advanced. The combination of CO_2 or HCO_3 with hemoglobin offers a simpler description.

We wish to thank Dr. D. D. Van Slyke and Dr. W. C. Stadie for their friendly and helpful criticism of this paper before publication.

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A MANOMETRIC MICROMETHOD FOR DETERMINA-
TION OF CARBON IN ORGANIC COMPOUNDS

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The organic material is subjected to wet combustion with a
mixture of chromic, sulfuric, and phosphoric acids in a tube at-
tached to the chamber of the Van Slyke-Neill (1924) manometric
apparatus. The carbon dioxide formed is drawn over into the
chamber and absorbed with dilute alkali solution. The unab-
sorbed gases are ejected, and the CO_2 is set free by acidification and
is measured manometrically, as in the determination of CO_2 in
blood.

The method is in principle a refinement of one already published
by Backlin (1930), who developed it for microdetermination of
blood lipids, the amounts of which he measured by the CO_2 they
evolved on wet combustion. Backlin used the original "volumet-
ric" type of blood gas apparatus (Van Slyke, 1917) and Nicloux's
(1927) silver chromate-sulfuric acid oxidizing mixture. In the
method as here presented the accuracy of the CO_2 measurement
is increased 5- to 10-fold by using the manometric gas apparatus,
the time required for combustion is cut down by utilizing a more
powerful oxidizing mixture, and details of technique have been
evolved which increase the certainty of the procedure.[1]

[1] A relatively unmodified adaptation of Backlin's method to the mano-
metric apparatus was described by Peters and Van Slyke ((1932), p. 433).
The present description contains changes and additions to the technique
which had not been evolved at the time of that publication.

Details of preparation of the different fractions of the plasma lipids for
microdetermination by this combustion will be published later, in connec-
tion with a study of the plasma lipid changes in Bright's disease.

Preliminary work by one of the writers (Kirk) has shown that this com-
bustion procedure may serve also for microdetermination of phosphoric
acid precipitated as the strychnine molybdate compound, and of sulfuric
acid precipitated as benzidine sulfate.

An analysis can be completed in 10 minutes from the time the combustion is begun till the CO_2 measurement is finished.

The method is applicable to amino acids, proteins, carbohydrates, lipids, and other non-volatile organic substances.[2]

Apparatus

The apparatus, of which two types are shown in Figs. 1 and 2, consists of a 15 cc. Pyrex combustion tube, A, the manometric gas apparatus, and a connecting tube, V, fitted to the combustion tube by a glass joint.   It is convenient to have a number of combustion tubes ground to fit the glass stopper of the same connecting tube.   The latter may be of soft glass, as it does not have to be heated.   It has several small bulbs, as in Fig. 1, or one larger bulb, as in Fig. 2, to condense any vapor that may distil from the heated combustion mixture.   The dropping funnel, M, shown in Fig. 2, is an accessory needed only when the laboratory air is likely to be contaminated with CO_2.   We have never found it necessary, but will describe its use for conditions when precautions must be taken against variations in atmospheric CO_2 content.

For microanalyses of samples containing 0.2 to 0.6 mg. of carbon the usual manometric apparatus is used (Fig. 1), and the pressures are measured with the gases at 2 cc. volume.   The results are usually accurate to 1 part in 200.   This type of analysis has been used in routine estimations of lipids   It can be employed to advantage when samples can be measured by volume as aliquot parts of solutions, but are too small to be weighed with accuracy on the microbalances now generally available.

When greater accuracy is desired, or when the samples must be weighed individually on a microbalance, the manometric chamber is changed by enlarging its upper bulb from 2 cc. to 10 cc. capacity (Fig. 2).   With this apparatus samples containing 1 to 3 mg. of carbon can be analyzed, and the accuracy is usually within 1 part in 500.   This apparatus is well adapted to precise elementary analysis.

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[2] For volatile substances the method requires modification.   Introduction of a small bulb with a heated platinum spiral into the connecting tube, V, has given fairly good results, but the details require further perfection before the accuracy is equal to that obtained with the routine procedure for non-volatile substances.
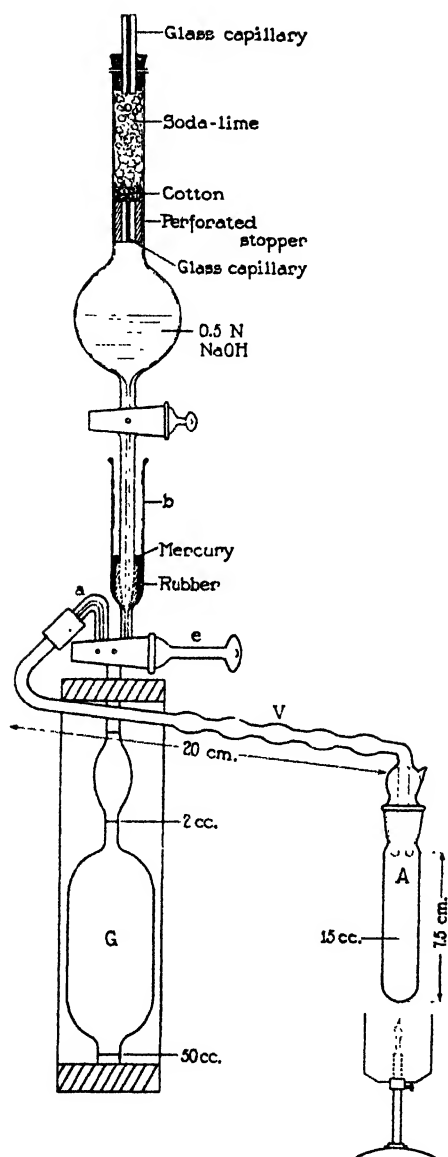
FIG. 1. Apparatus for combustion by Procedure A

For holding alkali solution CO_2-free, and for transferring portions of it to the chamber of the manometric apparatus, it is convenient to arrange a soda-lime tube as shown in Fig. 1, where the tube is seen in position ready to deliver alkali into the chamber. In place of this tube one may use a burette, guarded with soda-lime at the top, and equipped below with a tip long enough to reach to the bottom of the cup, b. The tip in either case is provided with a rubber ring to fit into the bottom of the cup as shown in Fig. 1. When the soda-lime tube or burette is not in use, its tip is kept immersed in mercury, to prevent absorption of CO_2 by the alkali at the outlet.

Heat for combustion is provided by an ordinary microburner protected from drafts by a mica shield such as is used with Welsbach burners. The shield and the burner are placed below the combustion tube in the positions indicated by Fig. 1.

Reagents

Combustion Fluid—125 cc. of concentrated sulfuric acid, 125 cc. of syrupy phosphoric acid, and 15 gm. of Merck's Extra Fine chromic acid are mixed in a scrupulously clean glass-stoppered flask, and shaken until the chromic acid is dissolved.

Approximately 0.5 N Sodium Hydroxide Solution of Minimal CO_2 Content—This is prepared from a stock solution of such high concentration that carbonate precipitates from it. To make the stock solution, c.p. sodium hydroxide is dissolved in an equal weight of water, cooled, and transferred to a paraffin-lined stoppered bottle, where the carbonate is permitted to settle to the bottom.

The 0.5 N solution is made with water which immediately before has been freed of CO_2 by addition of 1 drop of 0.1 N HCl per 100 cc. and boiling. To 100 cc. of this water 3 cc. of the clear, carbonate-free, concentrated alkali are added. The solution is drawn at once into a soda-lime protected burette, or the soda-lime tube shown in Fig. 1. Or, for future use, the solution may be poured into 100 cc. flasks which are immediately closed with vase-lined stoppers.

Approximately 1 N Lactic Acid[1]—c.p. concentrated lactic acid (specific gravity 1.20) is diluted to 10 volumes with water.

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[1] Lactic rather than a mineral acid is used to set free the CO_2 absorbed by the alkali in the chamber of the gas apparatus. Occasionally it appears

Approximately 5 N Sodium Hydroxide—30 cc. of the concentrated alkali solution described above are diluted to 100 cc. with water.

Procedure A. Without Preliminary Removal of CO_2 from Air in Combustion Tube

For this analysis the funnel M (Fig. 2) is not necessary. In our laboratory we have found this procedure adequate, even for elementary analysis in which a precision of 1 part in 500 was sought.

Preparation of Samples—The samples may be either weighed on a microbalance, or measured as aliquot portions of solutions in water, alcohol, petroleum ether, or other volatile solvent. In the latter case a measured volume of the solution is placed in the detached combustion tube and is evaporated to dryness by heating the open tube in a water bath. To remove last traces of alcohol, after the material is apparently dry a few drops of water are added and the tube is again heated to dryness in the water bath. Similar treatment with water is necessary to free from organic solvent phosphatides precipitated from ether solution by addition of acetone and calcium or magnesium chloride. The removal of organic solvent must be complete, or error will arise from combustion of the solvent itself. After an organic solvent has been evaporated from a sample, it is desirable to remove the combustion tube from the hood where the evaporation has taken place, and draw a little fresh air through the tube to remove any traces of solvent vapors. The tube is cooled to room temperature before the combustion is begun. The combustion should be performed in a room where the air is free from organic vapors.

When the sample is obtained as a precipitate, *e.g.* digitonin cholesteride, the precipitation and washing may be performed in the combustion tube, and the precipitate freed of organic solvents by heating as above described.

Measurement of Alkali Solution into Manometric Chamber— 2 cc. of the 0.5 N alkali are run into chamber G as follows. The

that a little SO_2 is formed in the combustion tube and absorbed by the alkali. This would be set free and in part extracted and measured with the CO_2 if a stronger acid were used to acidify. Lactic acid, however, is too weak to free SO_2 to a significant extent, and its use avoids the possibility of error from this source.

chamber is filled with mercury and 1 or 2 cc. of the mercury are run up into the cup, b, above the chamber, to serve as a seal for the tip of the soda-lime tube. The latter is pressed into the bottom of the cup under the mercury, as shown in Fig. 1. The mercury leveling bulb attached to G (but not shown in the figures) is placed level with the lower part of G, and alkali is admitted into G until the meniscus between mercury and alkali solution falls to the 2 cc. mark. The soda-lime tube is then removed, and the mercury in the cup is run down into the chamber, except for a drop left to seal cock e. The cup is washed out with water to remove traces of alkali.

Combustion—The connecting tube, V, is joined glass-to-glass, by means of a piece of best quality, fresh, heavy red nitrometer rubber tubing, to the capillary outlet, a, of the manometric chamber, as shown in Fig. 1.

The ground glass neck of the combustion tube is encircled with a ring of the combustion fluid, to make a tight joint. Then 1 cc. of the chromic acid combustion fluid is quickly measured into the combustion tube, and the latter is immediately attached to the ground glass joint of the connecting tube, before there is any opportunity for escape of CO_2, which may begin to form even in the cold fluid.

Cock e is turned to connect the combustion tube with the manometric chamber and the mercury in the latter is lowered enough to leave the upper third of the chamber free. The leveling bulb is lowered to the necessary point and left there, with the cock connecting it and the chamber open. Then, when the air in the combustion tube expands with heat, displacement of mercury from the chamber into the leveling bulb can occur.

The combustion fluid is heated to gentle boiling with a shielded microburner (*eyes should be protected by goggles*). 3 minutes heating at this temperature suffices for burning of the more easily oxidized substances, such as sugar, cholesterol digitonide, or amino acids, whereas 5 minutes may be required for cholesterol or fatty acids. Four or five times during the combustion the mercury leveling bulb is lowered and raised in order to draw gas from the combustion tube into the chamber. The combustion tube is shaken at intervals to insure contact of the sample and the combustion fluid, and to mix with the main body of fluid any drops that have spattered on the wall of the tube.

Absorption of Main Portion of CO_2 by Alkali in Gas Chamber—As soon as the combustion is complete, and while the mixture in still hot, the mercury in the chamber G is raised and lowered in the broad part of the chamber ten times to draw the CO_2 from the combustion tube into the alkali-containing chamber. The CO_2 drawn over is absorbed by the layer of alkali on the mercury and by the alkali film on the wall of the chamber. The mercury is finally lowered to the bottom of the chamber, and cock e is turned to close off the chamber from the combustion tube, which, however, is still left connected in position.

Ejection of First Fraction of Unabsorbed Gas—The greater part of the unabsorbed gas in the chamber is now ejected through cock e and the cup b as follows.

With cock e closed the leveling bulb connected with G is raised so that the mercury surface in it is 1 or 2 cm. above the mercury surface in G. The gas in G is put under slight pressure. The cock between leveling bulb and G is then closed, and the bulb is hung at a level such that the mercury surface in it is a little above e. Cock e is then turned to connect G and b, and mercury is admitted into G from the leveling bulb, driving out the air through e. Ejection of air is stopped while a small bubble still remains under e.

Drawing Over Last Traces of CO_2 from Combustion Tube to Gas Chamber—Cock e is sealed with a drop of mercury and the mercury in G is lowered to the middle of the chamber. Cock e is turned to connect the chamber again with the combustion tube A and the mercury in G is lowered to the bottom, thus reducing the pressure in A and G to about 40 mm. To draw the last trace of CO_2 over into the chamber, the mercury in G is raised and lowered five times more. Cock e is then closed, and the combustion and connecting tubes A and V are disconnected from the chamber. The curved capillary inlet, a, of the chamber is sealed with a drop of mercury drawn into it from a small mercury-filled test-tube.

Ejection of Last Portion of Unabsorbed Gas—This ejection is carried out as above described for the first portion of gas, except that the ejection is now approximately complete. During the ejection mercury is admitted carefully into G until the alkali solution rises just to the bottom of cock e. A little air remains in the capillary between G and b. Complete ejection of the air is not necessary: it contains no CO_2, and the small residual bubble in the capillary will not affect the analysis. It is, however, essen-

tial that no alkali solution should rise into cock b, to be lost, or to absorb CO_2 from the outside air.

Determination of CO_2—To set free the CO_2, 1.5 cc. of 1 N lactic acid are exactly measured into the manometric chamber from a 1.5 cc. stop-cock pipette, or from a fine burette capable of being read to 0.02 cc.   The admission is made in the manner shown in Fig. 3 of a former paper (Van Slyke, 1927).   It is necessary that the 3.5 cc. of solution in the apparatus during extraction of the CO_2 be measured accurately, because in calculation of the factors of Table 1 allowance is made for the proportion of CO_2 that remains dissolved in that volume of solution when equilibrium is reached in the extraction.   An error of $+0.1$ cc. in this volume of solution makes an error of -0.2 per cent in the CO_2 obtained for the pressure reading.   (There is, of course, a film of washing solution on the walls of the chamber at the beginning of each analysis, which adds its volume to that of the 3.5 cc. of measured solution.   The effect of this slight added volume appears to be balanced with approximate exactness by the effect of the sodium lactate in depressing somewhat the solubility of CO_2 in the water.)

After the lactic acid has been measured into the chamber the mercury is lowered to the 50 cc. mark and the chamber is shaken for a minute and a half to extract the CO_2 gas.   The gas volume is then reduced to the 2 or 10 cc. mark, with the usual precautions for CO_2 determination (see Van Slyke and Neill, 1924, p. 533, or Peters and Van Slyke, 1932, pp. 277-278).   The p_1 reading is checked by lowering the mercury again to the 50 cc. mark, shaking the chamber for 20 or 30 seconds, and bringing the gas again to 2 or 10 cc. volume.   This is repeated unless the p_1 readings agree within 0.3 mm.

After the p_1 reading has been made the cock between the chamber and its leveling bulb is opened, so that the gas in the chamber is at slight negative pressure.   The CO_2 is then absorbed by 5 N sodium hydroxide admitted from the cup (see Van Slyke and Neill, 1924, p. 546, or Peters and Van Slyke, 1932, bottom of p. 284).   In the analyses of 0.2 to 0.6 mg. of carbon, 0.3 cc. of the 5 N alkali is added.   When 1 to 3 mg. of carbon are determined 0.5 cc. of the alkali is used.   After absorption is complete the meniscus is returned to the 2 or 10 cc. mark and the p_2 reading is taken.

Blank analyses should be made daily, the combustion and other details being carried out as above described. The $p_1 - p_2$ reading obtained in the blank analysis is the c correction.

After each analysis the chamber is washed with dilute lactic acid and water, as after determination of blood CO_2 (see Van Slyke and Neill, 1924, p. 534; Peters and Van Slyke, 1932, p. 236).

Calculation

$$P_{CO_2} = p_1 - p_2 - c$$

$$\text{Mg. } CO_2 \text{ in sample} = P_{CO_2} \times f_{CO_2}$$

$$\text{``} \quad \text{carbon `` ``} = P_{CO_2} \times f_C$$

$$\text{``} \quad \text{lipid `` ``} = P_{CO_2} \times f_{\text{lipid}}$$

The factors f_{CO_2}, *etc.*, for use when P_{CO_2} is read with the gas at 2.000 cc. volume are given in Table I.

When the volume is other than 2.000 cc., as found in calibration of the chamber, the factors used are those in Table I multiplied by $0.5a$, where a is the precise volume at which the gas pressures are measured. If a is exactly 10 cc. the factors in Table I are multiplied by 5. It is convenient to compute the factors at intervals of $1°$ or $2°$ of temperature, for the type of analysis and room temperature range to be used, and to plot a curve of the factors on large scale coordinate paper, for convenience in interpolating the exact factors to within $0.1°$.

The factors for CO_2 in Table I are taken from Table IX of Van Slyke and Sendroy (1927). The factors for carbon are obtained by multiplying the CO_2 factors by 12/44. The factor for each lipid is obtained by multiplying the carbon factor by 100 ÷ (per cent C in lipid). The carbon contents are: cholesterol, 83.9 per cent; palmitic, stearic, and oleic acids, 75.0, 76.0, and 76.6 per cent respectively; tripalmitin, tristerin, triolein, 75.9, 76.8 and 77.4 per cent. The mean carbon content for the above three free fatty acids, which chiefly comprise animal fats, is 75.9 per cent, and of the fats 76.7, the maximum deviations being within ±1 per cent of these values; hence the percentages 75.9 and 76.7.

The factor for mixed plasma lipids is an approximate one estimated by assuming, as does Bloor (1929), that the mixture con-

sists of 2 parts of fat to 1 of cholesterol. The mixed lipid factors can be used when the cholesterol is mixed with either the neutral fats or with the fatty acids obtained after saponification, as in Stoddard's method, since the factors for neutral fats and fatty

TABLE I

For Calculation of Carbon Dioxide, Carbon, and Lipids from P_{CO_2} Obtained at 2 Cc. Volume after Wet Combustion of Organic Substances

Temper- ature	Factors by which P_{CO_2} is multiplied to give						
	CO_2	C	Cholesterol	Cholesterol when the digitonide is oxidized	Fatty acids	Neutral fat	Mixed plasma lipids
°C.	mg.	mg	mg.	mg.	mg	mg.	mg
15	0.005406	0 001474	0.001756	0 000579	0 001941	0 001921	0 001866
16	374	66	47	76	31	11	56
17	345	58	37	72	21	01	47
18	317	50	28	69	11	0 001890	36
19	287	42	18	66	00	80	26
20	262	35	10	63	0 001891	71	17
21	234	27	01	60	81	60	07
22	206	20	0 001692	57	71	51	0 001798
23	179	12	83	54	61	41	88
24	153	05	74	52	52	32	79
25	127	0 001398	66	49	42	23	71
26	102	91	58	46	33	14	62
27	078	85	50	44	25	06	54
28	054	78	42	41	16	0 001797	45
29	030	72	35	39	08	89	38
30	007	65	27	36	0.001799	80	29
31	0 004985	59	20	34	91	72	21
32	963	53	13	31	84	65	14
33	942	48	06	29	76	57	07
34	0 004922	0 001342	0 001599	27	0.001769	50	0.001700

acids differ by only 1 per cent. For calculation of cholesterol $C_{27}H_{46}O$, from the carbon determined in digitonin cholesteride, $C_{27}H_{46}O \cdot C_{55}H_{94}O_{38}$, which has 61.91 per cent of carbon and 24.3 per cent of cholesterol (Windaus, 1909), the carbon factor is multiplied by $100/61.91 \times 0.2431 = 0.3927$.

Nature of the c Correction

This correction, for CO_2 obtained in the blank analysis, is derived from three sources: (1) the slight amount of atmospheric CO_2 in the approximately 15 cc. of air in the combustion tube at the beginning of the analysis, (2) a trace of CO_2 in the sodium hydroxide solution (the procedure for removal of carbonate in preparing the solution is not absolutely complete), (3) CO_2 from the 1 cc. of combustion fluid. With the air and reagents of our laboratory the magnitude of these three components is indicated by the following figures.

Pressure of CO_2 from 2 cc. 0 5 N NaOH. .	5 5	mm.	at 2	cc.	volume
" " " " air in combustion tube	5 2	"	" 2	"	"
Pressure of CO_2 from 1 cc. combustion fluid.....	3 3	"	" 2	"	"
Total c correction	14 0	"	" 2	"	"

When the pressure is measured with the gas at 10 instead of 2 cc. volume the c correction is only one-fifth of the above.

We had anticipated the possibility that, in the portion of the blank due to CO_2 from air in the combustion tube, significant variations would occur during a working day from variations in the CO_2 content of the laboratory air, and that it might be necessary to fill this tube with CO_2-free gas before each analysis. In fact, however, this factor was found to be so nearly constant during an ordinary working day, that the only precaution necessary with respect to it, for the degree of accuracy aimed at in this analysis, has been ordinarily good ventilation of the room.

In case, however, it should be necessary to carry out the analyses in a room where the air has a high or fluctuating CO_2 content, the effect of atmospheric CO_2 can be eliminated as described below for Procedure B.

Procedure B

To exclude errors from variations in the CO_2 content of the laboratory air, the CO_2 of the atmospheric air in the combustion tube is removed before the combustion is begun.

Preparation of Sample—If the sample is in solution the solvent is removed as described for Procedure A.

Removal of CO_2 from Atmospheric Air in Combustion Tube—
2 to 3 cc. of the CO_2-free 0.5 N sodium hydroxide are admitted into
the chamber and the special connection tube (Fig. 2), furnished
with a stop-cock dropping funnel, *M*, is attached to the capillary
outlet of the chamber. The stop-cock of the dropping funnel *M*,
and the neck of the combustion tube *A* are lubricated with syrupy
phosphoric acid. In attaching the combustion tube containing
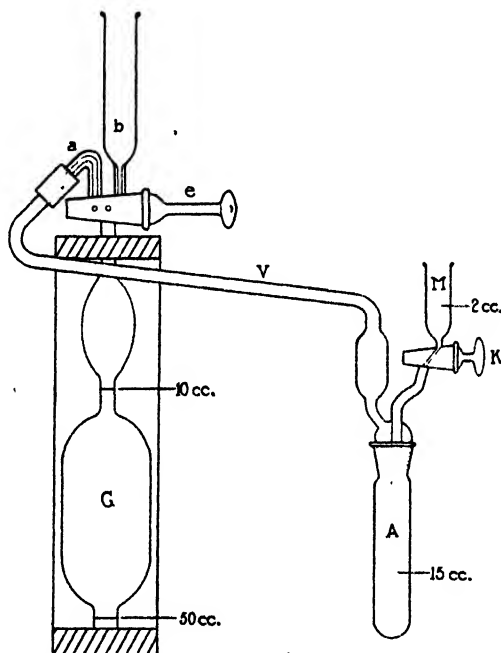
Fig. 2. Apparatus for combustion by Procedure B

the sample, care is desirable to prevent drops of the lubricant from
running down the walls of the combustion tube and coming into
contact with the sample. *A* and *G* are connected by turning cock
e. The air from the combustion tube is then driven back and
forth ten times between the tube and the chamber, so that all the
CO_2 in the air is absorbed by the alkali solution in the chamber.
The air is finally returned to the combustion tube. The alkali
is completely ejected from the chamber and is replaced by 2 cc.
of fresh alkali, measured exactly into the chamber from a burette.

(A burette, protected from atmospheric CO_2 by soda-lime at the top, is used instead of the uncalibrated soda-lime tube in Fig. 1, when a chamber with a 10 cc. bulb at the top is used, as shown in Fig. 2.)   It is unnecessary to wash the chamber before admission of the second portion of alkali; the amount of CO_2 in the film of the first portion left adhering to the wall of the chamber is negligible.

Combustion—Cock *e* is again turned to connect the chamber with the combustion tube.   1 cc. of combustion fluid is measured into

TABLE II

Results of Analyses by Procedure A

Substance	Sample weight	Combustion time	P_{CO_2} at 2.011 cc. volume	Temperature	Factor	Carbon		Deviation from theoretical
						Found	Theoretical	
	mg	*min*	*mm*	*°C*		*mg.*	*mg*	*per cent*
Galactose	1 094	3	307.7	22 0	0 001428	0 4391	0 4373	+0 41
"	1 094	3	311 8	25 7	1402	0.4372	0.4373	-0 02
"	1 094	3	313 7	26 0	1400	0 4391	0.4373	+0 41
Glycine	1 556	3	350 5	22.0	1428	0 5005	0 4976	+0 58
"	1 556	3	349 7	22 6	1423	0 4976	0.4976	±0 00
"	1 556	3	351 8	22.5	1424	0 5010	0 4976	+0 68
Palmitic acid	0 6833	5	362 1	24 7	1408	0 5098	0.5125	-0.53
" "	0 6833	5	362 6	24.9	1407	0 5100	0 5125	-0 49
Cholesterol	0.2310	5	136.5	24 0	1413	0.1929	0 1938	-0 46
"	0 3213	5	196 8	29.0	1380	0 2716	0 2696	+0 74
"	0 4090	5	251 7	31 0	1366	0 3438	0 3432	+0 17
"	0 6426	5	387 8	28 5	1383	0 5363	0.5392	-0 54
"	0 8180	5	495 9	29 6	1376	0 6824	0.6863	-0 57
" formate	0 2735	5	157 2	25 6	1402	0 2204	0.2213	-0 40
" "	0.5471	5	315 8	25 1	1406	0 4437	0 4426	+0 25

M and admitted to the combustion tube, followed by a small bubble of air.   The bore of the stop-cock *K* is sealed with combustion fluid, and the leveling bulb is placed in such a position as to leave the upper one-third of the chamber free of mercury.   The reduction of pressure in *A* expands the air bubble under cock *K* and draws down into *A* the residue of chromic acid solution in the inlet tube below *K*.   The combustion fluid in *A* is heated, and the analysis from this point on is carried out as in Procedure A.

Precaution—The cup of the dropping funnel *M* must be guarded against dust by an inverted tube, which is removed only during the moment when the combustion fluid is placed in the funnel.

Tables II and III indicate the nature of the results obtained. The galactose analyzed was a recrystallized preparation of Merck's c.p. product. The glycine and cholesterol were Kahlbaum's. The cholesterol formate was prepared as described by Page and Rudy (1930). The palmitic acid was purified by recrystallization

TABLE III

Results of Analyses of Galactose Samples Containing 1 to 3 Mg. of Carbon and with CO_2 Pressures Measured at 9.971 Cc. Volume

The combustion time in each case was 2.5 minutes.

Sample weight	P_{CO_2} at 9.971 cc. volume	Temperature of P_{CO_2} reading	Factor	Carbon		Deviation from theoretical
				Found	Theoretical	
mg.	*mm.*	*°C.*		*mg.*	*mg.*	*per cent*
3.527*	201.8	24.4	0 006989	1.4104	1.4098	+0.04
3.527*	202.1	25 0	0 006970	1.4087	1.4098	−0.08
7.054*	406.2	26.0	0 006935	2 8170	2.8196	−0 09
7.047*	405.2	25 6	0.006949	2.8144	2 8167	−0.08
6.042†	354.7	30.5	0.006790	2 4084	2.4150	−0.27
5.098†	300.4	31.0	0.006775	2 0352	2 0377	−0.12
5.624†	334.0	31.9	0 006748	2 2538	2 2479	+0.26

* Sample measured as an aliquot of a solution prepared by weight.

† Sample weighed out on microbalance.

from methyl alcohol. The substances, except for the samples in Table III which are designated as weighed on the microbalance, were all dissolved in suitable solvents, and aliquot portions were measured out with calibrated pipettes into the combustion tubes, where the solvents were volatilized before the combustion.

Carbon in organic substances is determined by wet combustion in a tube attached to the chamber of the manometric gas apparatus. The CO_2 evolved is absorbed by alkali solution in the chamber, and is then determined by acidification, extraction, and

manometric measurement of the gas. An analysis requires about 10 minutes. The accuracy is of the order of 1 part in 200 with 0.2 to 0.6 mg. of carbon and the usual manometric chamber used for blood gases. With samples of 1 to 3 mg. of carbon and a manometric chamber which brings the gas to 10 instead of 2 cc. for pressure measurement, the accuracy is of the order of 1 part in 500.

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COMPARISON OF GASOMETRIC, COLORIMETRIC, AND TITRIMETRIC DETERMINATIONS OF AMINO NITROGEN IN BLOOD AND URINE

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Of the titrimetric, colorimetric, and gasometric methods now available for determination of amino acids, none is entirely specific. Consequently it has been impossible to tell what proportion of the "amino acid nitrogen" determined in blood or urine with any one of these procedures really represents the amino acids, and what proportion is due to other substances capable of reacting with the reagents used. However, if it can be shown that methods based on several different reactions yield similar results for preexisting amino acids in blood and urine, and afford accurate measurement of added known amounts, it may be assumed with a reasonable degree of probability that the effect of interfering substances is not important. The desirability for a study of the question presented itself to the writers when one of them (Kirk), using the gasometric nitrous acid method, observed regularly in uremic coma a several fold increase in the amino nitrogen of the blood, whereas the numerous clinical studies appearing in recent years with the Folin (1922, *a*) colorimetric method had disclosed no such phenomenon.

The methods chosen for the present study are the formaldehyde titration of Sørensen (1907), the colorimetric method of Folin (1922, *a*, *b*), and the nitrous acid gasometric procedure of the authors (Van Slyke, 1929; Van Slyke and Kirk, 1932), each applied to both blood and urine, and the acetone titration of Linderstrøm-Lang (1928) applied to blood as described by Zirm and Benedict (1931).

Zirm and Benedict appear to be the only previous authors who have reported comparative results on blood or urine by different methods. They analyzed four blood filtrates by their application of the acetone titration, by the Folin colorimetric method, and by

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the gasometric method of Van Slyke (1929). Results of the same order of magnitude (6 to 8 mg. of amino N per 100 cc. in three normal bloods and 9 to 11 mg. in one leucemic blood) were obtained with all three procedures, but the gasometric method in the three normal bloods gave 0.6 to 1.0 mg. more than the other analyses. In applying the gasometric method Zirm and Benedict apparently omitted the preliminary removal of urea prescribed by Van Slyke (1929). The effect of this omission in blood of ordinary urea content would be to raise the apparent amino nitrogen content by 0.5 to 1.0 mg. per 100 cc., the extent by which Zirm and Benedict's gasometric values exceed their titration results. Alanine added to blood by Zirm and Benedict was accurately determined by the acetone titration, but not by the colorimetric method.

Schmidt (1929) and Re and Potick (1930) also found that the colorimetric method failed to determine completely amounts of amino nitrogen greater than those ordinarily found in human blood. Hiller and Van Slyke (1922) found that amino acids added to blood were accurately determined by the gasometric method.

The different methods do not yield identical results with all the amino acids derived from proteins. Further, with either titration method applied to a diamino or dicarboxylic acid, the result obtained when the starting point is the pH spontaneously assumed by a solution of the pure amino acid (its isoelectric point) is altogether different from the result obtained when the starting point is arbitrarily adjusted to a given pH. The difference in the behavior of the different types of amino acids towards the reagents used for their determination, and, in the titrations, the further differences introduced by preliminary pH adjustment, appear never to have been summarized. Without such a summary it would be difficult to know what concordance is theoretically possible among results of the various methods. We shall therefore precede our experimental report with a résumé of the principles of the titrations, the effects upon them of preliminary pH adjustments, and of the behavior of the reagents used for the colorimetric and gasometric determinations.

Theory of Amino Acid Titrations

Most monoaminomonocarboxylic acids have similar titration curves, which vary but slightly from that given in Fig. 1. There

are in each such amino acid two buffer groups, with pK' values[1] in the neighborhood of 2 and 9.5, respectively (Harris, 1923–24; Kirk and Schmidt, 1929). Which buffer group represents the amino group and which the carboxyl is at present not entirely certain, and for defining the titration behavior it is not necessary to know which is which. We shall, therefore, for this discussion, simply characterize the two groups as the first, or lower, with pK'_1 = about 2, and the second, or upper, with pK'_2 = about 9.5. For dicarboxylic and diamino acids, with three buffer groups each, the pK' values will similarly be indicated in order from least to greatest as pK'_1, pK'_2, and pK'_3.

The necessity of using such devices as addition of acetone, alcohol, or formaldehyde in order to make amino acid titrations practicable is indicated by the curves of Figs. 1 to 5. In simple water solution titration of the group with lowest pK' would, in each type of amino acid illustrated, involve use of an end-point of about pH 1.5, while titration of the groups with the highest pK' values would require end-points of pH 11 or higher. At pH 1.5 the correction for the free HCl necessary to give this reaction becomes an important part of the total HCl utilized in titrating a dilute amino acid solution; and the curve at this point is so steep that accuracy in fixing the end-point is difficult. Furthermore, such end-points as pH 1.5 and 11 are outside the

[1] The symbol pK' is used in the sense employed by one of the writers in a discussion of buffers (Van Slyke, 1922). It is the pH of the buffer solution when the buffer group, if acid, is half neutralized by strong alkali, or, if basic, is half neutralized by strong acid. In the case of an acid buffer, such as acetic acid, $pK' = -\log K'_a$, where K'_a is the apparent acid dissociation constant. In the case of a basic buffer, $pK' = -\log \dfrac{K_w}{K'_b}$, where K'_b is the apparent basic dissociation constant and K_w is the dissociation constant of water, approximately 10^{-14}. When pK' is thus defined *a strong acid is one with a low pK'*, while *a strong base is one with a high pK'*.

The use of pK' in this sense was found to be advantageous in discussing the general behavior of buffers. In the present connection it has a particular advantage in that for a curve with a given location the pK' has the same value whether the buffer group is basic or acidic. When the buffer groups are characterized by pK' values in this sense, the curves can be discussed without assumption as to whether, in Fig. 1 for example, the upper curve represents the NH₂ group and the lower the carboxyl group, or *vice versa*.

practicable range of indicators, which, being buffers themselves, are applicable only between pH 2 and 11, and preferably between 3 and 10.   To make the amino acids titratable with indicators, devices have therefore been introduced to change either the media in which they are dissolved or the structures of the amino acids themselves in such a manner that at least one of the buffer groups has its pK' moved within the range between 5 and 8, so that titrations can be carried out with end-points not lower than pH 3 nor higher than pH 10.

In the titration methods of Foreman (1920), of Willstätter and Waldschmidt-Leitz (1921), and of Linderstrøm-Lang (1928), the solvent is changed to one of lower dielectric constant by addition of 10 or more volumes of alcohol or acetone.   Alcohol, used by Foreman and by Willstätter and Waldschmidt-Leitz, moves pK'_2 down far enough to bring its buffer group within the zone practicable for titration with alkali.   Acetone, used by Linderstrøm-Lang, moves pK'_1 up far enough to make its buffer group titratable with HCl (see Fig. 1).[2]

Sørensen (1907), in the first and most widely applied titration method, added formaldehyde, which, like alcohol, lowers the upper pK'.   In this case the change is not in the dielectric constant of the medium, but in the structure of the amino acids.   Schiff (1899, 1901, 1902) showed that formaldehyde reacts with primary and secondary aliphatic amines as indicated by the equations, $RNH_2 + OCH_2 = RN:CH_2 + H_2O$ and $2R_2NH + OCH_2 = (R_2N)_2CH_2 + H_2O$. (The latter reaction occurs with imino groups in proline, oxyproline, and perhaps histidine.)   The resulting condensation products, $RN:CH_2$ and $(R_2N)_2CH_2$, are much weaker bases than the original amines; and their upper titration curves are shifted towards the left when plotted as in Fig. 1.   Sørensen applied the reaction to amino acids, and found that addition of formaldehyde moved the second pK' down to the neighborhood of pH 7, so that this buffer group could be titrated with NaOH to pH 9 with phenolphthalein

[2] Presumably alcohol and acetone, which have similar dielectric constants (26 and 21 respectively compared with 81 for water), have nearly like effects on both types of buffer groups in the amino acids.   It happens that conditions for titration in alcohol have been worked out for the upper group and in acetone for the lower.

as indicator, or in some amino acids more completely to pH 10 with thymolphthalein.

Nature of Buffer Groups Titrated

Since titration of a monoamino acid in water solution with HCl leads to the lower titration curve with its mid-point near pH 2 (Fig. 1), it was at first natural to assume that this curve was that of the basic NH_2 group directly neutralized by the added HCl. And similarly that titration with NaOH, leading to the curve with mid-point near pH 9.5, represented the course of neutralization of the carboxyl group by the alkali. This assumption was made in the earlier work of Sørensen and others, and must still be admitted as a possible explanation of the observed titration curves. In accordance with it, however, the NH_2 group would have to possess an extremely weak basicity, similar to that of aniline, and the carboxyl group a very weak acidity, similar to that of phenol. An equimolar mixture of phenol and aniline would give a complete titration curve almost identical with that of glycine in Fig. 1. In the isoelectric zone between pH 4 and 7 such weakly basic and acidic substances would be free from salt-like combination, either with other acids or bases or with each other. If the acid and basic groups of an amino acid are of this weak type the state of the amino acid in the isoelectric zone is represented by the ordinary structural formula, $HOOCRNH_2$, with no charges or other evidence of electrolyte behavior of either the NH_2 or COOH group.

Bjerrum (1923), however, has pointed out that it is improbable that aliphatic amino and carboxyl groups should be so weakly basic and acidic, respectively, as is required by the above hypothesis. Aliphatic amines in general are strong bases, with high pK' values resembling that of ammonia, which is about 10. And aliphatic acids have pK' values mostly between 3 and 5, and therefore much nearer the pK'_1 than the pK'_2 values of the monoamino acids. Bjerrum believes therefore that in an amino acid of the type of glycine (Fig. 1) the pK'_1 is that of the carboxyl group, the pK'_2 is that of the amino group, and that the amino acid has in the isoelectric state the character of an internal ammonium salt of an unusually strong organic acid. This internal salt he represents by the formula, $^-OOCRNH_3{}^+$, and terms it a zwitter ion, since it carries in itself the charges of both a positive and a negative ion. In accordance

with this explanation, when glycine is titrated with HCl the latter sets free the carboxyl group from this salt combination, as HCl sets free lactic acid from ammonium lactate (Fig. 6), and the hydrogen ions in the titrated glycine solution are those liberated by dissociation of the carboxyl H in $HOOCCH_2NH_3Cl$. The accordance of this explanation with the facts is shown by the similarity of the titration curves of glycine (Fig. 1) and ammonium lactate (Fig. 6). The accordance extends also to the similarity of behavior of lactic acid and the lower buffer group of glycine in the acetone titration, and to the similarity of behavior of ammonia and the upper buffer group of glycine in the formaldehyde titration (Figs. 1 and 6). The fact that the upper buffer group of glycine is the one to have its pK' chiefly affected by reaction with formaldehyde is also readily comprehensible in accordance with the hypothesis that this group is the NH_2 group. It appears more probable that the formaldehyde condensation affects chiefly the basicity of the NH_2 group that is changed to $N:CH_2$ rather than the acidity of a carboxyl group not altered by the condensation. Other evidences favoring the zwitter ion theory have been advanced by Borsook and MacFadyen (1930).

The alcohol titration appears not yet to have been applied to blood or urine, but the formaldehyde and acetone titrations have found application under conditions which it appears desirable to examine in some detail, in order to ascertain the significance of the results and the possible sources of error.

Titration Curves of Amino Acids

The types of titration curves given in water solution by the different types of amino acids and by ammonium lactate are represented in the solid curves of Figs. 1 to 6. The titrations in the presence of acetone and formaldehyde are indicated, as accurately as available data permit, by the dash line curves. Acetone in the case of each amino acid increases the lowest pK', which according to Bjerrum represents a carboxyl group. Formaldehyde on the other hand lowers the uppermost pK', and in diamino acids the intermediate pK' also, which according to Bjerrum represent the reacting NH_2 and NH groups. In consequence the titration curves of the affected buffer groups are shifted to the right or left as indicated by the arrows.

The locations of the dash line curves are only approximately exact. The correct curve may in each case be somewhat to the right or left of that given. The reason for the lack of more certainty is that the only data available for these curves are the titration end-points reported by Sørensen and Linderstrøm-Lang for amino acids in formaldehyde and acetone solutions. Near the end-points the curves are flat, and interpolations are consequently
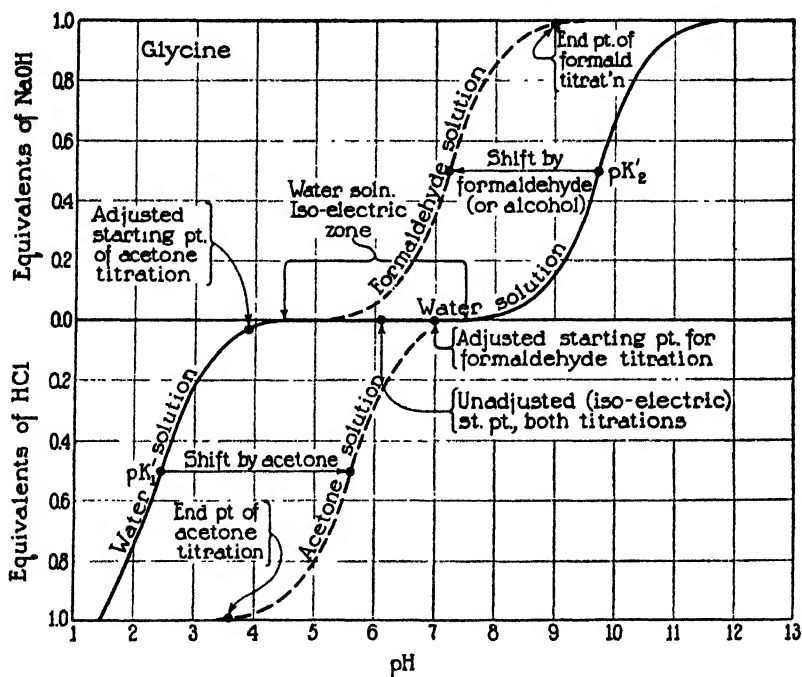
Fig. 1. Titration curve of glycine. Water solution data from Harris (1923-24). Formaldehyde curve estimated from end-points at pH 9 to 10 given by Sørensen (1907). Acetone curve estimated from titration results of Linderstrøm-Lang.

inexact. If the pH values at the mid-points of each curve in these solutions were available the locations could be made accurately, but such data are not yet available. The end-point data nevertheless serve to locate the curves with sufficient accuracy to indicate the quantitative behavior of the different types of amino acids in the titrations.

The water solution curves in the extreme pH zones below 1 and above 13 deviate in shape from the typical S curves given by buf-
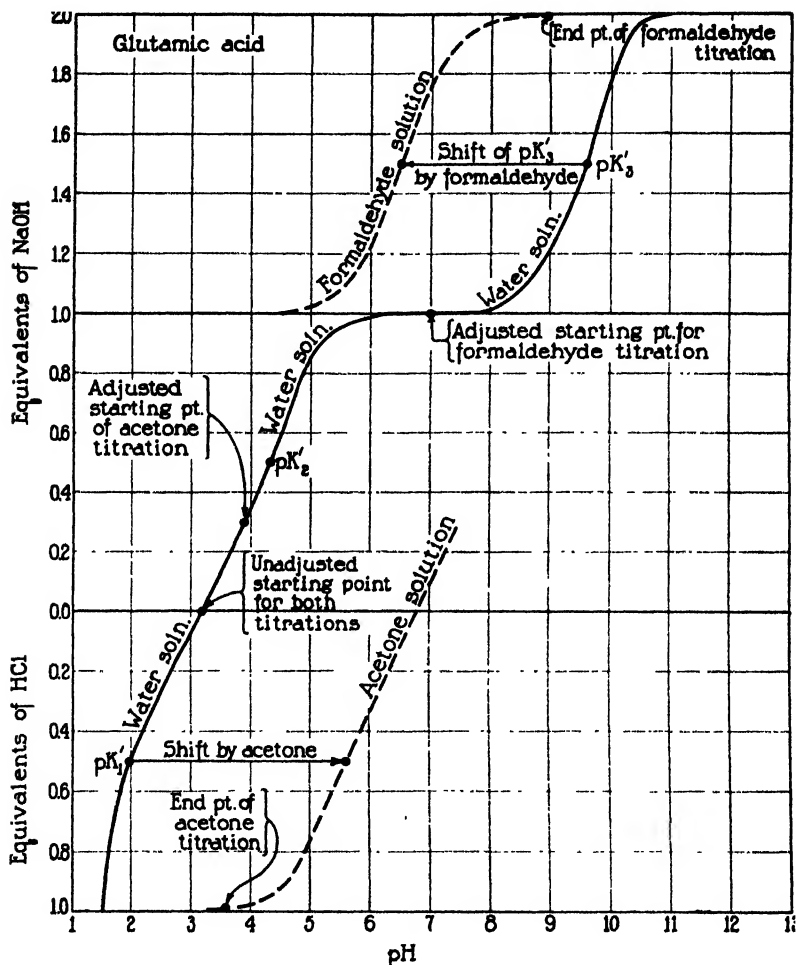
FIG. 2. Titration curve of glutamic acid. Water solution data from Kirk and Schmidt (1929). Formaldehyde curve estimated from end-points of Sørensen. Acetone curve estimated from titration results of Linderstrøm-Lang.

fers nearer neutrality, because in the end-zones the H^+ and OH^- become quantitatively important parts of the total ion concentra-

tions.   The nature of the effect has been discussed in connection with Equation 30 of Van Slyke's paper on buffers (1922).   In Figs.
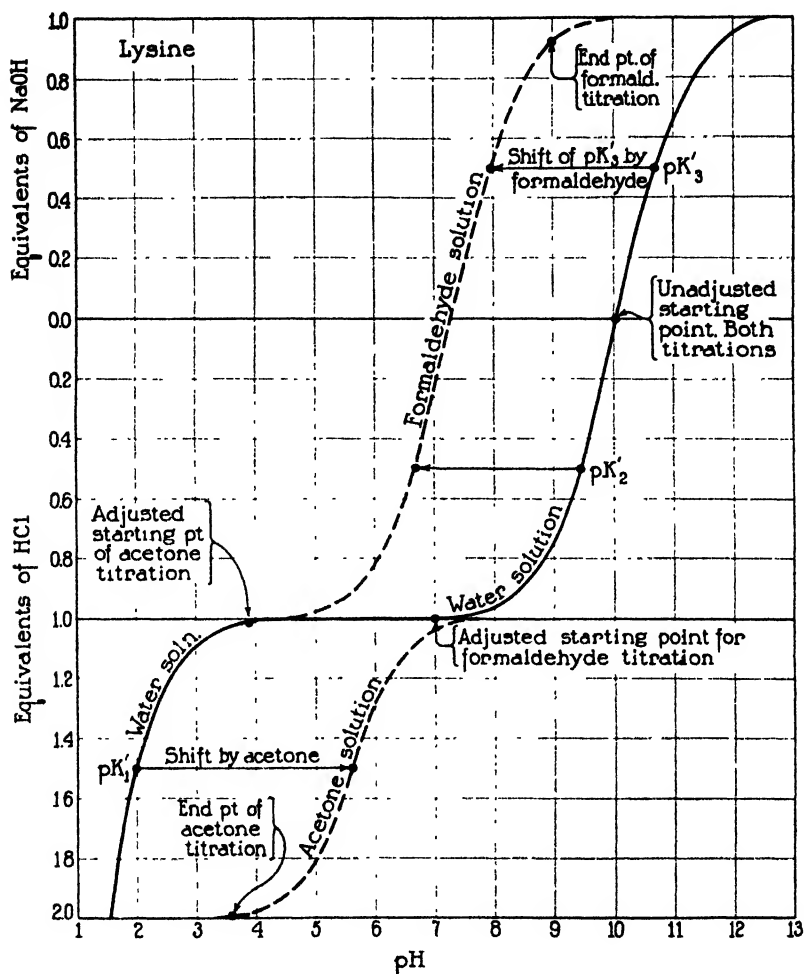
FIG. 3. Titration curve of lysine. Water solution data from Tague (1920) as corrected by Harris (1923-24). Formaldehyde curve estimated from end-points given by Sørensen (1907). Acetone curve estimated from titration results of Linderstrøm-Lang.

1 to 6 the curves in these zones are calculated by this equation for 0.1 molecular concentrations of the amino acids.

Acetone Titration

From the Isoelectric Point (Unadjusted Starting Point)—In the Linderstrøm-Lang titration addition of 10 or more volumes of ace-
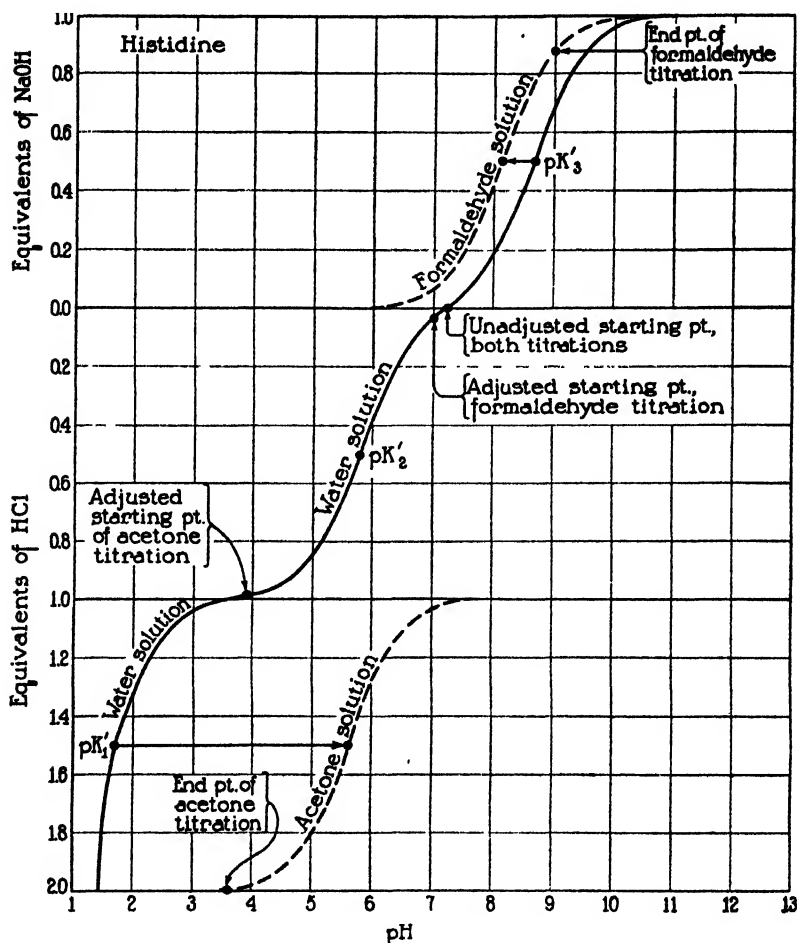
Fig. 4. Titration curve of histidine.   Water solution data from Harris (1923–24).   Formaldehyde curve estimated from end-points of Sørensen (1907).   Acetone curve estimated from titration results of Linderstrøm-Lang.

tone to the water solution of an amino acid raises the pK'_1 value so far above its aqueous level that its buffer group can be quanti-

tatively titrated with HCl to an end-point of approximately 3.6. (HCl is added to the acetone-treated solution until the color of the naphthyl red indicator (benzene-azo-α-naphthylamine) equals that
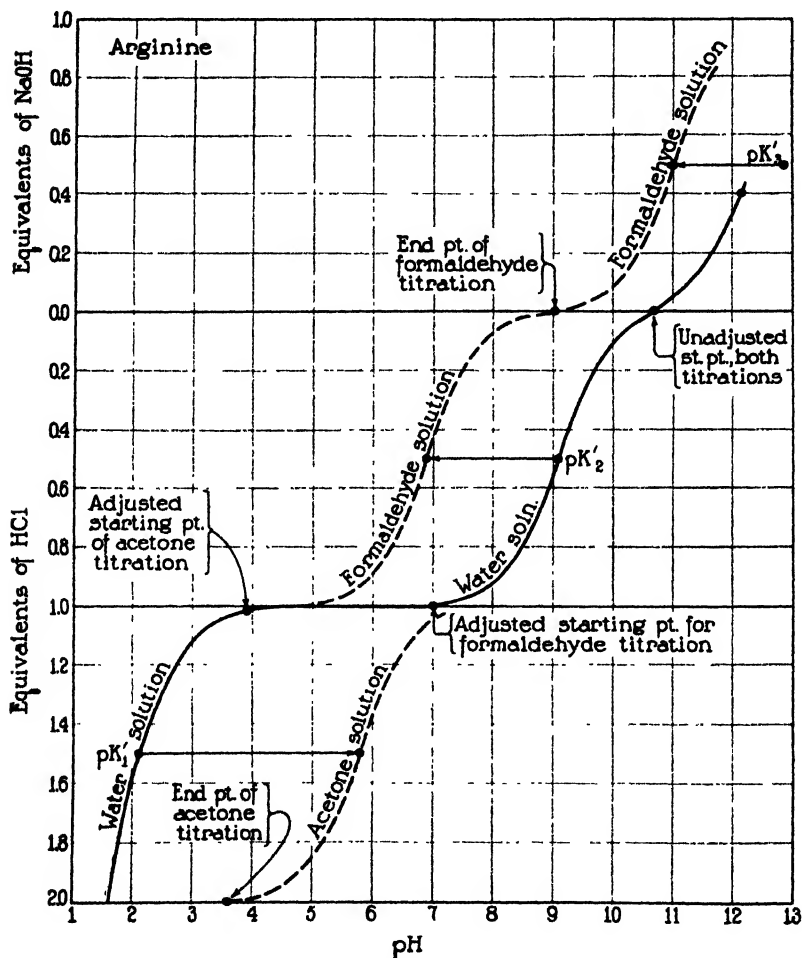
FIG. 5. Titration curve of arginine. Water solution data from Hunter and Borsook (1924). Formaldehyde curve estimated from end-point of Sørensen (1907). Acetone curve estimated from titration results of Linderstrøm-Lang.

in a control water-acetone solution containing HCl in about 0.00024 N concentration, equivalent to pH 3.6 if the HCl is com-

pletely dissociated.)   This procedure was shown to yield theoretical results with pure amino acids when no preliminary adjustment of the pH was made, the starting point of the titration being in each case the isoelectric point of the amino acid.   Under this condition each monoamino acid neutralizes 1 equivalent of HCl and each diamino acid 2 equivalents (see Figs. 1 to 5, HCl required
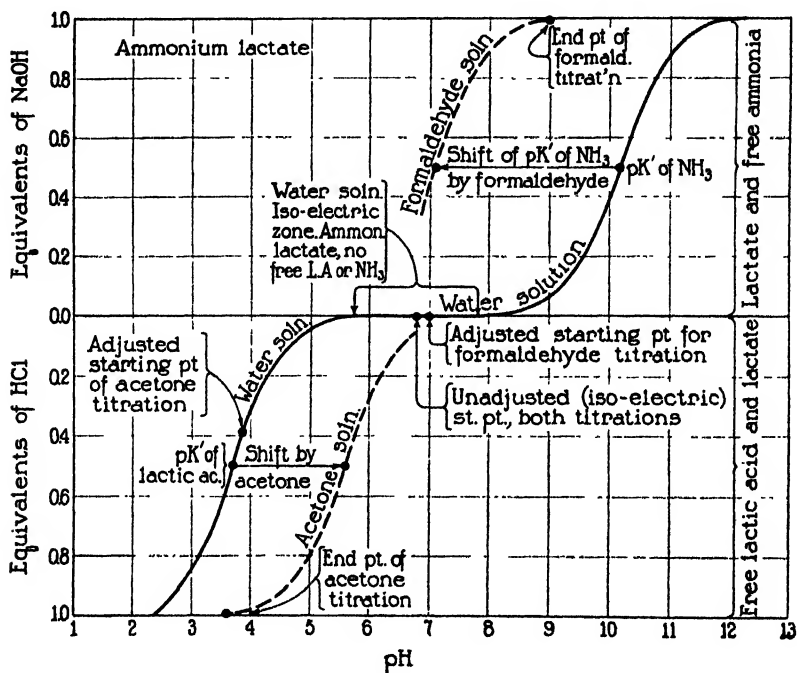
Fig. 6. Titration curve of ammonium lactate.   Water solution curve calculated from pK' values of lactic acid (Van Slyke, Sendroy, Hastings, and Neill, 1928) and of ammonia (Noyes, Kato, and Sosman, 1910).   Formaldehyde curve estimated from end-points of Sørensen (1907).   Acetone curve from authors' titration results.

between unadjusted starting points and end-points of acetone titration).

From Starting Point Adjusted to pH 3.9—In order to adapt the titration to blood filtrates Zirm and Benedict (1931) introduced a preliminary adjustment of the aqueous solution to pH 3.9 before the acetone was added.   It appears that no better fixed starting

point for the titration could have been chosen. It is the one point on the curves, not only of the monoaminomonocarboxylic acids (Fig. 1), but also of the diamino acids (Figs. 3 to 5) from which in every case an exact, or almost exact, equivalent of HCl is required to react completely with the first buffer group. After acetone addition each amino acid of these two classes requires 1 equivalent of HCl per mol for titration, the difference between mono- and diamino acids being eliminated by the preliminary pH adjustment. In the case of the dicarboxylic acids, exemplified by glutamic acid in Fig. 2, the adjustment is not quite so fortunate. The pH 3.9 point falls at a point on the middle curve which is reached by neutralizing from 0.3 to 0.5 of the second buffer (carboxyl) group with added alkali. Consequently the amount of HCl required after acetone addition to titrate back this alkali (0.3 equivalent in the case of glutamic acid) is added to the 1 equivalent required for the lowest group. To obtain an approximate mean titration value of 1 equivalent of HCl for each amino acid in a mixture of all those derived from proteins, the starting point used by Zirm and Benedict seems nevertheless to be the best that could be chosen.

Its necessary use, however, exposes the method to error by partial inclusion of any non-amino organic acids that may be present in the mixtures titrated. The addition of acetone shifts upwards the pK' values of such acids so far that they become neutral to naphthyl red (see experimental part of this paper for demonstration with lactic and acetic acids). The carboxyl groups are affected by acetone in the same manner as the first buffer groups of the amino acids (a fact which again makes it easy to believe with Bjerrum that the first buffer group is the carboxyl). If to a water solution of an organic acid, such as lactic, enough alkali is added to raise the pH to 3.9, and then acetone is added and HCl titration carried out as in Linderstrøm-Lang's determination, an amount of HCl is used equivalent to the alkali added in the preliminary adjustment. Therefore non-amino organic acids titrate more or less completely as amino acids by the Zirm and Benedict technique, the extent to which they thus titrate being measured by the amount of alkali required to bring each one to the pH 3.9 of the starting point. The fraction of an equivalent of alkali bound by any buffer acid at pH 3.9 can be calculated from the pK' by Henderson's equation (see Henderson's graph in Fig. 9 of Van Slyke (1922), or

in Fig. 90, p. 893, of Peters and Van Slyke (1931)). Thus by calculation acetic and hydroxybutyric acids, with pK' about 4.7 each, bind 0.14 equivalent of alkali; acetoacetic acid with pK' of 3.8 (Henderson and Spiro, 1909) binds 0.56 equivalent; and lactic acid with pK' of about 3.7 (Van Slyke, Sendroy, Hastings, and Neill, 1928) binds 0.62 equivalent. Therefore, if these organic acids are present, they will be included as amino acids in the Zirm and Benedict titration to the extent of approximately 12, 14, 56, and 62 per cent respectively of their molar concentrations. Experimental results in this paper with lactic and acetic acids accord with these calculations.

In the dicarboxylic amino acids (Fig. 2) the second carboxyl group of each behaves in the same manner as an added organic acid. In these three amino acids, glutamic, hydroxyglutamic, and aspartic, the second carboxyl group has pK' values given respectively as 4.2, 4.2, and 3.96 (Kirk and Schmidt, 1929; Hopfield *et al.*, 1920). They therefore bind at pH 3.9 approximately 0.33, 0.33, and 0.46 equivalents of alkali, and will theoretically require in the acetone titration by Zirm and Benedict's technique 1.33, 1.33, and 1.46 equivalents of HCl respectively (for behavior of glutamic acid see Fig. 2). Experimentally the writers have obtained 1.36 and 1.5 equivalents for glutamic and aspartic acids. Because of the strong buffer effects of both at pH 3.9 the preliminary adjustment to this point does not take a sharply defined amount of alkali, and the difference between the results obtained and those theoretically calculated is within the limit of error of the former.

The effects of Zirm and Benedict's insertion of a preliminary adjustment to pH 3.9 before the acetone titration may be summarized as follows: (1) The titration of monoaminomonocarboxylic acids is unaffected; (2) the diamino acids titrate with 1 instead of 2 equivalents of HCl each; (3) the dicarboxylic acids titrate with 1.3 to 1.5 instead of 1 equivalent of HCl; (4) non-amino organic acids are included in the titration to an extent measured by the amount of alkali which they bind at pH 3.9. This exceeds 0.5 equivalent per mol in the case of acids as strong as lactic and acetoacetic. It is evident that the Zirm and Benedict application of the acetone titration is valid for amino acids only in the absence of significant amounts of other organic acids.

Of substances other than amino and organic acids, Linderstrøm-

Lang showed that about 1 per cent of urea nitrogen is measured as amino acid nitrogen in the acetone titration. Ammonium chloride and sulfate are not included, and do not interfere. The titration curve of ammonia is at so much higher a pH range than that of the groups included in the acetone titration that the latter is not affected (see Fig. 6).

Sørensen's Formaldehyde Titration

From the Isoelectric Point (Unadjusted Starting Point)—The theory of this titration has already been discussed, and the results with the different types of amino acids and ammonia can be deduced from Figs. 1 to 6. Although it appears to be the basicity of the NH_2 or NH group that is really affected by formaldehyde in each case, yet the location and behavior of the different büffer groups is such that the average amount of alkali used in the titration, when its starting point is the isoelectric point of the amino acid, is more nearly 1 equivalent for each carboxyl group than 1 for each basic nitrogen. Thus, in the dicarboxylic acids (Fig. 2) the extra carboxyl group (with pK'_2) neutralizes, independently of the action of formaldehyde, 1 equivalent of NaOH in the titration. To this is then added another equivalent, utilized after formaldehyde addition by the uppermost buffer group (presumably the NH_2), so that the total alkali required is 2 equivalents. In the three diamino acids, for reasons most easily deduced from Figs. 3 to 5, relations are such that 1 equivalent of alkali is used per mol, except in the case of arginine, where none is used. Except for arginine, therefore, in the formaldehyde titration with unadjusted starting points approximately 1 equivalent of alkali is used for each amino acid carboxyl group present.

Ammonia and amines in the free state are, after treatment with formaldehyde, so nearly neutral to phenolphthalein that they are practically without effect on the titration.

From Starting Point Adjusted to pH 7—This preliminary adjustment was introduced by Henriques (1909) for urine titrations and by Northrop (1926) for biological fluids in general. Such adjustment has different effects on the different types of amino acids as follows:

The *monoaminomonocarboxylic acids* (Fig. 1) are unaffected. Their isoelectric zones cover the pH range 4 to 7 (Harris, 1923–24;

Kirk and Schmidt, 1929), so that the amount of acid or alkali required to adjust this type of amino acid to pH 7 is practically negligible.

The *dicarboxylic acids* (Fig. 2) titrate with 1 instead of 2 equivalents of alkali. The pK'_2 value of each of these amino acids is about 4. Consequently, to bring its water solution to pH 7 requires addition of a complete equivalent of alkali. The uppermost buffer group ($pK'_3 = 9 - 10$) then behaves, after formaldehyde addition, exactly like the upper buffer group of a monocarboxylic amino acid, and only 1 equivalent of alkali is used in the final titration.

In the *diamino acids*, lysine and arginine, adjustment to pH 7 requires the addition of 1 equivalent of HCl (Figs. 3 and 5), and therefore increases by 1 equivalent the amount of alkali required in the final titration. Histidine (Fig. 4) has an isoelectric point at practically pH 7, so that it acts, either with or without preliminary adjustment, like a monoamino acid, and requires 1 equivalent of alkali in the titration in either case.

Aliphatic amines of the types RNH_2 and R_2NH, when brought to pH 7 form salts of the types $RNH_2 \cdot HCl$ and $R_2NH \cdot HCl$. On subsequent formaldehyde addition their basicities are so weakened (pK' values diminished) that they set free the HCl for titration. Ammonia behaves in a similar way, with formation of tetramethylene amine (Fig. 6).

The effect of preliminary neutralization of an amino acid mixture on the results of the formaldehyde titration may be summarized by stating that such neutralization changes the titration from an approximate measure of the carboxyl groups to an approximate measure of the primary NH_2 groups plus the NH groups of the proline and oxyproline, together with any ammonia and free amines that may be present.

The results are not sharply stoichiometric in all cases. In proline, oxyproline, and histidine the shift of the pK' of the upper buffer group by formaldehyde appears to be not so great as in other amino acids. Consequently, when the titration is carried out with pH 9 as end-point, the results with proline and oxyproline are only about 80 per cent quantitative, and with histidine about 88 per cent (Sørensen, 1907; Iodidi, 1918). In tyrosine, on the other hand, the amount of alkali required is a few per cent above 1 equivalent, because the phenol hydroxyl neutralizes a little alkali at pH 9.

The Folin (1922, a, b) Colorimetric Method—A colored product is formed by reaction of the amino acids with sodium naphthoquinone-β-sulfonate, and the intensity of the color serves as a measure of the concentration of amino acid. The colored compounds appear not to have been isolated, nor the reaction identified by which they are formed, but the reaction appears to be with the amino groups. Ammonia is stated by Folin to be an interfering substance. In determinability of the nitrogen of the individual amino acids the method appears to approximate the formaldehyde titration, but to be somewhat less sharply quantitative.

Gasometric Method (Van Slyke, 1911, 1912, 1929)

The amino acids react with nitrous acid, $RNH_2 + HNO_2 = N_2 + ROH + H_2O$, and the N_2 set free is measured. In the 3 to 4 minutes time required for complete reaction by the α-amino acids, ammonia reacts to the extent of about 25 per cent and urea to 7 per cent of its nitrogen. Hence both these substances must be removed if present in interfering amounts.

Summary of Results Yielded by Different Methods with Different Types of Amino Acids

From the summary given in Table I it appears that the two titrations, when used with adjusted end-points, give approximately the same results with all the amino acids except the following: The three dicarboxylic and the two pyrrolidine acids, each measures only about 80 per cent as much by the formaldehyde as by the acetone titration. Lysine, on the other hand, measures 200 per cent as much by the formaldehyde as by the acetone titration. It appears that in mixtures of the amino acids such as are likely to result from breakdown of proteins no great difference is to be expected from the results by the two methods. The 100 per cent difference of lysine in one direction would be more or less balanced by the 20 per cent differences of 5 other amino acids in the opposite direction.

The gasometric method gives approximately the same results as the formaldehyde titration, except that proline and oxyproline give zero reaction with the gasometric reagents. One may therefore expect the gasometric method to give results markedly different from those of the titrations only when proline and oxyproline make an important part of the amino acid mixture. They do

TABLE I

*Behavior of Different Types of Amino Acids in Acetone Titration,
Formaldehyde Titration, and Gasometric Determination*

The figures given are calculated per mol of amino acid.

Amino acids and interfering substances		Acetone titration		Formaldehyde titration		Gasometric NH₂ determination	Total N atoms in molecule
Type	Individuals	From isoelectric point (unadjusted starting point) to pH 3.6	From starting point adjusted to pH 3.9 to end-point at pH 3.6	From isoelectric point (unadjusted starting point) to end-point at pH 9	From starting point adjusted to pH 7 to end-point at pH 9		
		eq. HCl	*eq HCl*	*eq NaOH*	*eq. NaOH*	*N atoms*	
Monoamino-monocarboxylic acids	Glycine, alanine, valine, leucine, tryptophane, tyrosine, phenylalanine, serine, cysteine	1	1	1	1	1	1 except tryptophane with 2
Monoaminodicarboxylic acids	Glutamic, hydroxyglutamic, aspartic acids	1	1.3–1 5	2	1	1	1
Pyrrolidine acids	Proline and oxyproline	1	1	0 8	0.8	0	1
Diamino acids	Arginine	2	1	0	1	1	4
	Histidine	2	1	1	1	1	3
	Lysine	2	1	1	2	2	2
Interfering substances	NH₄OH	1	0	0	1	0.25	1
	Urea	0.01	0.01	0	0	0.14	2
	Lactic acid	0	0.6	1	0	0	0
	Hydroxybutyric acid	0	0.14	1	0	0	0
	Acetoacetic acid	0	0.6	1	0	0	0

so in the hydrolytic products of gelatin, and it will be seen later
(Table V) that after gelatin feeding the increased amino acid excre-
tion measured gasometrically is definitely less than that measured
by the formaldehyde titration.

Behavior of Lactic and Acetic Acids in Acetone Titration

Standards—In the experiments here described Zirm and Bene-
dict's Standards I and II are used.

Standard I is made by diluting 1 cc. of 0.025 N HCl to 200 cc.
with water.   2 cc. of this solution are mixed with 4 drops of a 0.1
per cent alcoholic solution of naphthyl red.   The HCl concentra-
tion is 0.000125 N, and the pH therefore approximately 3.9.   When
the preliminary adjustment of the unknown is nearly finished to
the color of this standard, the latter is diluted by adding enough of
the 0.000125 N HCl to bring its volume up to that of the unknown.

Standard II is prepared by mixing 2 cc. of water, 0.3 cc. of
0.025 N HCl, 4 drops of 0.1 per cent naphthyl red solution, and 30
cc. of acetone.   The pH is 3.6 if the activity coefficient of the H^+
ions is unity.   The color, however, because of the effect of the
acetone on the pK' of the indicator, is that of a water solution of
pH about 4.8.

Lactic Acid Titration without Preliminary pH Adjustment—To
2 cc. of 0.1 N lactic acid were added 4 drops of naphthyl red.   The
solution assumed the deep red of a markedly acid reaction.   Then
30 cc. of acetone were added, and the color changed to the yellow
which the indicator assumes in neutral or alkaline water solutions.
To change to the slightly acid color of Standard II, 0.08 cc. of 0.1
N HCl was required.   This amount is, within the limit of titration
error, equivalent to the 0.3 cc. of 0.025 N HCl used in preparing
Standard II.   The lactic acid, in the presence of the acetone,
therefore behaved as a neutral substance to the indicator.

Lactic Acid, with Preliminary pH Adjustment to 3.9—To 2 cc. of
0.1 N lactic acid 4 drops of naphthyl red were added, then 0.1 N
sodium hydroxide until the color was that of Standard I.   The
amount of 0.1 N alkali required was 1.2 ± 0.1 cc. in repetitions of
the experiment.   The preliminary end-point was not sharp, be-
cause lactic acid is a good buffer at this pH.   To the solution thus

titrated to pH 3.9, 30 cc. of acetone were added. The color changed to the clear alkaline yellow. Alcoholic 0.1 N HCl was then added until the color equaled that of Standard II. The amount required, corrected for the free HCl in Standard II, was 1.2 ± 0.1 cc., equivalent to 60 ± 5 per cent of the lactic acid present. In the discussion of the principle of the acetone titration we have calculated that the amount titrated in this method would be 62 per cent.

Acetic Acid without Preliminary pH Adjustment—This behaved precisely as the lactic acid. The acidity of the carboxyl group towards the indicator used was depressed to zero by addition of acetone.

Acetic Acid with Preliminary pH Adjustment—2 cc. portions of 0.1 N acetic acid were treated like the lactic acid in the corresponding experiment. A volume of 0.3 ± 0.02 cc. of 0.1 N sodium hydroxide was taken to bring the acetic acid to the color of Standard I, and the same volume of 0.1 N alcoholic HCl, within the limit of titration end-point error, was required after acetone addition to titrate back to the color of Standard II. An average of 0.15 cc. equivalent of 0.1 N hydrochloric acid was therefore used for each mol of acetic acid, approximating the 0.14 equivalent calculated in the discussion of the principle of the acetone titration.

Blood and Urine Analyses

The methods were applied as described below. It will be noted that in preparing the blood filtrates colloidal iron is used for the two titrations and the gasometric analysis, while tungstic acid is used in preparing the filtrate for the colorimetric determination, in order to adhere to Folin's conditions. Hiller and Van Slyke (1922), however, have found that when a mixture of amino acids from hydrolyzed protein was added to blood the added amino nitrogen could be quantitatively recovered in filtrates prepared by either method.

Acetone Titration. Blood—The Linderstrøm-Lang method was applied to blood filtrates as described by Zirm and Benedict (1931), except that in preparing the blood filtrates we used less colloidal iron than the latter authors.

5 cc. of whole blood, without addition of anticoagulants, were diluted with 35 cc. of distilled water and heated to boiling in an

Erlenmeyer flask.   1 cc. of a 10 per cent solution of "colloidal iron oxide" was added, a few drops at a time, and the mixture was shaken after each addition.   The clear supernatant fluid separated readily, and showed no protein when tested with sulfosalicylic acid.   The mixture was transferred to a Pyrex tube, made up with boiling water to a volume of 52 cc. (= 50 cc. at 20°), and immediately filtered. The filtrate was cooled to room temperature, and 15 cc. aliquots were concentrated to dryness on the steam bath.   Each residue was redissolved in 2 cc. of distilled water and the pH adjusted as described by Zirm and Benedict, by adding 0.025 N alcoholic HCl until the color changed to that of Standard I.   Then after addition of 30 cc. of acetone, the solution was titrated by adding more 0.025 N alcoholic HCl till the color became that of Standard II (the standards are described in the first paragraph under "Experimental").   As indicator 4 drops of 0.1 per cent alcoholic naphthyl red solution were used, in each standard and in the blood filtrate titrated.   A Bang microburette was used both in the preliminary pH adjustment and in the titration.   From the HCl used in the titration the amino nitrogen content of the blood is calculated.

$$\text{Mg. amino N in sample} = 14 \times 0\ 025 \times (A - 0\ 3)$$
$$\text{``   N per 100 cc. blood} = 23\ 3\ (A - 0.3)$$

A represents the cc. of 0.025 N hydrochloric acid used in the final titration.   The correction of 0.3 cc. is made for this amount of acid added to the standard to which the color of the titrated solution is brought.

An error of 0.01 cc. in the titration causes an error of 0.2 mg. of amino nitrogen per 100 cc. of blood in the result, and this represents the constancy with which titrations can usually be duplicated in a blood analysis.

Formaldehyde Titration.   Blood—The micro form of Sørensen titration described by Northrop (1926) was used.   The tungstic acid filtrate did not prove suitable for it, but the colloidal iron filtrate, prepared as described above, was satisfactory.

From each blood four 15 cc. portions of this filtrate, each equivalent to 1.5 cc. of blood, were concentrated to dryness and the residues were each redissolved in 2 cc. of water.   The standard colored solutions with neutral red and phenolphthalein for the starting point (pH about 7) and end-point (pH about 9) were prepared by

Northrop's technique from two of these portions of blood filtrate, so that any preformed color in the filtrate was compensated. The titrations were carried out in duplicate on the other two portions as described by Northrop, except that for the 2 cc. portions of solution 0.4 as much of each reagent was used as directed by Northrop for 5 cc. portions. From the volume of 0.01 N hydroxide used a correction was subtracted for the volume required to neutralize the amount of formaldehyde added. The calculation is

$$\text{Amino N in sample} = 0.14A$$
$$\text{"} \quad \text{"} \quad \text{per 100 cc. blood} = 9.34A$$

A represents the corrected cc. of 0.01 N alkali used in the final titration.

The titration error does not appear to exceed 0.02 cc. of 0.01 N alkali, equivalent to 0.2 mg. of amino N per 100 cc. of blood.

Formaldehyde Titration of Urine. Henriques' (1909) Method— Henriques' application of the Sørensen method was slightly modified to fit it to albuminous urines, and the ammonia was removed by vacuum distillation, instead of being determined and corrected for.

The proteins of nephritic urine were precipitated by heating on a steam bath after addition of 5 drops of 10 per cent acetic acid per 50 cc. of urine. The sample was then washed into a volumetric flask, phenolphthalein was added, and the urine phosphates were precipitated by addition of barium chloride in substance, followed by barium hydroxide sufficient to render the urine alkaline to the phenolphthalein. The mixture was then made up to volume with distilled water and filtered. ·A known volume of the alkaline filtrate was concentrated *in vacuo* to remove the ammonia, and was then acidified with hydrochloric acid and transferred to an Erlenmeyer flask by means of CO_2-free water. After neutralization to sensitive litmus paper (first end-point) freshly prepared neutral formaldehyde solution was added, and the solution was titrated with 0.2 N barium hydroxide till it attained the full red color of an alkaline standard.

Formaldehyde Titration of Urine (Northrop)—The procedure for removal of phosphate and carbonate was essentially the same as used in preparation of urine samples for the Henriques titration. To 50 cc. of urine in a 100 cc. volumetric flask were added 2 gm. of

barium chloride, which were allowed to dissolve by shaking. No phenolphthalein indicator was added, but the solution was made alkaline to litmus paper with saturated barium hydroxide, after which 5 cc. more of saturated barium hydroxide were added. The solution was made up to 100 cc. with distilled water, and filtered after standing for 15 minutes. 50 cc. of the urine filtrate were concentrated *in vacuo* till ammonia was removed. The residual solution was then acidified to litmus paper with 1 N HCl and distillation was continued to remove carbon dioxide. The sample was then transferred to a 50 cc. volumetric flask, neutralized approximately with CO_2-free 1 N NaOH, and made up to volume. (1 cc. of the final solution represented 0.5 cc. of urine.)

2 cc. samples of this solution were used for preparation of Northrop's alkaline end-point standard and for titration. For the neutral red starting point standard, however, 1 cc. of the original untreated urine diluted with 1 cc. of water was used, because if the barium-treated urine had been mixed with the phosphate buffer employed for this standard the phosphate would have been precipitated. The titration was performed as described for titration of the blood filtrates.

Gasometric Determination. Blood—The determination was carried out as previously described (Van Slyke, 1929), filtrate equivalent to 0.5 cc. of blood being used for each manometric analysis. The urea was in all cases destroyed in the whole blood with urease, and the ammonia formed was removed from the blood filtrate by boiling with magnesium hydroxide.

Gasometric Determination. Urine—The free amino acid nitrogen in urine was determined as described by Van Slyke and Kirk (1932), with preliminary removal of the urea with urease and of the ammonia by vacuum distillation.[3]

[3] *Modification of Gasometric Method for Glucose-Containing Urines*—If glucose is present extra precautions must be taken to prevent the urine from becoming alkaline during urease action, or the ammonia will combine with the glucose to form an amine which reacts slowly with nitrous acid, causing results to be too high. The following modification was devised some years ago. It was not used in any of the analyses reported in this paper, but should be employed if the gasometric method is applied to diabetic urines.

10 cc. of urine are placed in a 200 cc. measuring flask with 30 cc. of CO_2-charged water from a Seltzer bottle. 1 cc. of a 10 per cent solution of Squibb's urease is added, and the mixture is saturated with toluene. The

Colorimetric Analysis. Blood—The original method of Folin (1922, *a*) was followed.

The naphthoquinone reagent was prepared according to the details given by Folin. This preparation gave a deeper color and was capable of reacting with about twice as much amino nitrogen as was a purchased preparation. It is possible that the latter had deteriorated with age, as it had been about the laboratory for at least a year. Of the naphthoquinone reagent prepared in the laboratory, 10 mg. sufficed to react with 0.2 mg. of glycine nitrogen. This limit was sharp, however; adding indefinite amounts of amino nitrogen more than 0.2 mg. caused hardly any more color.

10 cc. of Folin-Wu filtrate of blood were used for each sample. 20 to 24 hours were allowed for the development of color, while the mixture of sample and reagent was kept in a dark closet at room temperature of about 23°. The standard and the unknown solutions were each made up to a volume of 25 cc. before comparison in a Duboscq colorimeter.

Colorimetric Analysis. Urine—The procedure of Folin (1922, *b*) was followed in all details. The standard solutions contained 0.1, 0.2, and 0.3 mg. of glycine nitrogen. The size of the urine samples was varied, so that they could be compared with different standards.

The results of the analyses are given in Tables II to V.

Results with Blood

The two *titration methods* agree with the *gasometric* as closely as could be expected, considering the fact that the different methods do not determine exactly the same amount of amino nitrogen in every amino acid (Table I). After addition of known amounts of

flask is stoppered and let stand overnight at room temperature. The next day add 80 cc. of water and, for each per cent of sugar present in the urine, 2.5 cc. of a 20 per cent solution of copper sulfate (20 gm. of $CuSO_4 \cdot 5H_2O$ per 100 cc.). Dilute to the 200 cc. mark and decant into a larger Erlenmeyer flask. Add powdered $Ca(OH)_2$ until the mixture is alkaline to phenolphthalein, mix thoroughly, and let stand ½ hour to precipitate all the glucose. Decant through a dry folded filter. Concentrate 100 cc. of the filtrate, either to dryness on a water bath, or to about 10 cc. *in vacuo* as described by Van Slyke and Kirk (1932). Acidify the residue with a few drops of glacial acetic acid and dilute to 25 cc. The analysis of 5 cc. portions and the calculation are as described by Van Slyke and Kirk (1932).

amino acid to blood quantitative recovery was obtained, both gasometrically and by both titrations (Table III).

TABLE II

Comparison of Colorimetric, Gasometric, and Titrimetric Amino Nitrogen Determination in Human Blood

In each colorimetric analysis 10 cc. of Folin-Wu filtrate were used, and the standard contained 0.07 mg. of glycine nitrogen.

Hospital No.	Colorimetric			Gasometric Amino N per 100 cc.	Acetone titration Amino N per 100 cc.*	Formaldehyde titration Amino N per 100 cc	Urea N per 100 cc blood
	Readings		Amino N per 100 cc.				
	Standard	Unknown					
	mm.	*mm.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
8349	20 0	25 2	5 6	8 7	7 4		10
	20 0	24 7	5 7	8 7	7 5		
8342	20 0	24 3	5 8	9 4	9.6		120
	20 0	21 9	6 4	9 3	10.5†		
8277	20 0	24 2	5 8	7 5	7.9		7
	20 0	26 0	5 4	7 8	8 0		
8322	20 0	26 5	5 3	8 5	7.2		18
	20 0	24 6	5 7	8 5	7.3		
8336	20 0	23 8	5 9	8.1	8 1		35
	20 0	24 2	5 8	8 2	8 2		
8395	20 0	24 2	5 8	13 1	11 1	11.8	22
	20 0	23 2	6 1	13 1	11 3	11.6	
8400	20 0	19 0	7 4	15 7	14 5	15 0	74
	20 0	18 0	7 8	15 7	14 6	14 9	
7872	20 0	29 0	4 8	12 4	14.3‡	10 9	85
	20 0	29 2	4 8	12.6	15 3	10.8	
7905				8.6	8 91		15
					17 96§		

* Corrected in each blood by subtracting 1 per cent of the urea nitrogen.

† End-point difficult.

‡ Preuremic. High figures for acetone titration may have been due to accumulation of organic acids.

§ After addition to the blood of 0.1 volume of 0.105 N lactic acid. The increase of 9 mg. per cent in the amino nitrogen found is equivalent to 0.64 mM of amino acid per liter of blood, which is 61.5 per cent of the concentration of added lactic acid.

The fact, that the acetone titration yields results in ordinary blood which agree as closely as they do with results by the formaldehyde and nitrous acid methods, indicates that the content of lactic

TABLE III

Recovery of Amino Acids Added to Human Blood

Material	Volume of blood represented in sample	NH_2-N in standard	Naphtho-quinone used	Colorimetric readings		Amino N found per 100 cc.	Recovery of added NH_2-N
				Standard	Unknown		

Folin colorimetric method

	cc.	mg.	mg.	mm.	mm.	mg.	per cent
Blood....	1	0 07	10	30	29 0	7 2	
" after addition of alanine (13 06 mg. NH_2-N per 100 cc. of blood)........	1	0 14	10	20	19 2	14 6	44.5
Same...	1	0 14	20	20	14 96	18 7	87.9
Blood after addition of alanine (26 11 mg. NH_2-N$_a^a$ per 100 cc. of blood)..	1	0 14	10	20	17 59	15.9	23 3
Same	1	0 14	20	20	10 48	26.7	74 6

Acetone titration method

Original amino nitrogen content of whole blood						9 93	
Found after addition of alanine (18.70 mg. NH_2-N per 100 cc. whole blood)						28 00	
Recovered........						18.07	96 6

Gasometric method

Original amino nitrogen content of serum						7 51	
Found after addition of alanine (26.18 mg. NH_2-N per 100 cc. serum). ....'						33 41	
Recovered..						25.90	99 2
Found after addition of mixed ammonia-free monoamino acids from casein hydrolysis (15.75 mg. NH_2-N per 100 cc. serum)..... .. . .|						23 44	
Recovered........						15 93	101 1

Formaldehyde titration method (Northrop)

Original amino nitrogen content of whole blood....						11.20	
Found after addition of glycine (16.43 mg. NH_2-N per 100 cc. whole blood)....						27.72	
Recovered............................						16.52	100 6

TABLE IV

Comparison of Colorimetric, Gasometric, and Formol Titration Determinations of Amino Nitrogen in Human Urine, and in Amino Acid Solutions of NH_2 Content Similar to That of Urine

Ammonia was removed by permutit for the colorimetric analyses, by vacuum distillation for the titration and gasometric analyses.

Material	Volume of solution or urine in sample	NH_2-N in standard	Colorimetric readings		Amino N per 100 cc.	Gasometric Amino N per 100 cc	Formaldehyde titration*
			Standard	Unknown			
	cc.	mg	mm.	mm	mg.	mg.	mg.
NH₂-free solution of mono-amino acids from hydrolyzed casein†	0 5	0 1	20 0	14 40	28 6	32 8	31 5
	1	0 2	20 0	16 06	24 9		
	2	0 3	20 0	15 00	20 0		
	5	0 3	20 0	13 00	9 2		
Alanine solution, 55.33 mg. per cent alanine, 8.70 mg. per cent NH₂-N	0 5	0 1	15 0	30 50	9 8	8 75	8 58
	1	0 1	20 0	21 95	9 1		
	2	0 2	20.0	23 48	8 5		
	3	0 3	20 0	23 47	8 5		
	4	0 3	20 0	18 00	8 3		
	5	0 3	20 0	14 85	8 1		
Normal urine, E. K.	0 5	0 1	20 0	17 26	23 2	18 3	19 0
	1	0 2	20 0	20 62	19 4		
	2	0 2	20 0	12 64	15 8		
	2	0 3	20 0	18 33	16 4		
	3	0 3	20 0	14 64	13 7		
Normal urine, A. A.‡	0 5	0 1	20.0	12 16	32.9	19 2	18 3
	0 5	0 2	20 0	24 68	32 4		
	1	0.2	20 0	14 90	26 8		
	1	0 3	20 0	22 14	27 1		
	2	0.3	20 0	15.00	20.0		
	3	0 3	20 0	14 20	14 1		
	4	0 3	20 0	13 20	11 3		
	5	0 3	20.0	13 50	8 9		
Normal urine, J. B.§	0.5	0 1	20.0	11 06	36 6	15 9	16 0
	0.5	0 2	20 0	21 30	37.5		
	1	0.2	20 0	11.30	35.4		
	1	0.3	20 0	16.80	35.7		
	2	0.3	20.0	12.20	24 6		
	3	0.3	20 0	11.40	17.6		
	4	0.3	20.0	11.40	13.2		

TABLE IV—*Concluded*

Material	Colorimetric					Gasometric	Formaldehyde titration*
	Volume of solution or urine in sample	NH₂-N in standard	Colorimetric readings		Amino N per 100 cc.	Amino N per 100 cc.	
			Standard	Unknown			
	cc.	mg.	mm.	mm.	mg.	mg.	mg.
Normal urine, V. K.§	0 5	0.2	20.0	25 70	31.1	16 3	19 2
	1	0.2	20 0	13 10	30 5		
	1	0 3	20 0	19.98	30 0		
	2	0 3	20 0	12 80	23 4		
	3	0 3	20 0	12 00	16 7		
	4	0 3	20 0	11 80	12 7		
Normal urine, J. M.	0 5	0 1	20 0	24 95	15 0	11 3	11 9
	1	0.1	20 0	12.25	16 3		
	1	0 2	20 0	25.14	16.4		
	2	0 3	20 0	24 62	12 2		
	3	0 3	20 0	17.90	11 8		
J. M. same urine + 26.0 mg. per cent alanine amino N	0 5	0 2	20 0	19 60	40 8		
	1	0 2	20 0	12 32	32 5		
	1	0 3	20 0	20 78	28.9		
	2	0 3	15 21	15 21	19 7		
Normal urine, A. A.						11 9	14 8
A. A. same urine + 32.2 mg. amino N as alanine						44 4	48 9

* In these formaldehyde titrations the preliminary pH adjustment was made with litmus, according to Henriques (1909).

† For each analysis 25 mg. of sodium naphthoquinone-β-sulfonate (5 cc. of 0.5 per cent solution) were used. This was sufficient to react with 0.5 mg. of amino nitrogen in the form of glycine.

‡ In this urine another set of colorimetric analyses, in which the ammonia was removed by vacuum distillation instead of by permutit, gave practically identical results.

§ In these analyses phenolphthalein was added to the standard and the urine samples, and enough sodium carbonate solution to the latter to make the colors identical (Schmitz and Scholtyssek, 1928).

and other non-amino organic acids is ordinarily slight. 1 mм per liter of lactic acid would add $0.6 \times 1.4 = 0.8$ mg. of amino N per 100 cc. to the value estimated by the acetone titration. The

amount of interfering organic acid present in *ordinary* human blood appears to cause no greater error than this.

With the *colorimetric* method the amino nitrogen values obtained in whole blood were only from 60 to 80 per cent as great as those by titration or gasometric methods.   When sufficient alanine was added to blood to raise the amino nitrogen content to levels such as have been gasometrically observed in uremia (20 to 30 mg. per cent) the increases in amino nitrogen, determined with the usual

TABLE V

Comparison of Amino Nitrogen Determination by the Gasometric Method and Formaldehyde Titration in Human Urines before and after Ingestion of Gelatin

Hospital No.	Gasometric	Formaldehyde titration	Remarks
	Amino N per 100 cc.	Amino N per 100 cc.	
	mg.	*mg.*	
Normal	11 5	10 9	Fasting
"	63 9	70 2	After ingestion of 62 gm. gelatin
7986	15 7	15 1	Proteinuria, fasting
7986	43 0	48 7	After 60 gm. gelatin
8101	4 7	3 6	Proteinuria, fasting
8101	21 3	21 9	After 62 gm. gelatin
Normal	23 2	25 4*	
"	24 5	25 1*	

* The preliminary adjustment to pH about 7 was made with neutral red as internal indicator by Northrop's technique.   In the other analyses the procedure of Henriques was followed, with preliminary adjustment with litmus paper.

conditions of the colorimetric determination, were only 23 to 44 per cent of the amounts added.   By doubling the amount of naphthoquinone reagent, the recovery was increased to 75 to 88 per cent (Table III).   It does not appear that the colorimetric method, routinely applied in its original form, can be expected to reveal blood amino nitrogen increases with even approximate accuracy.   Our conclusion in this regard agrees with that of Re and Potick (1930).

It appears possible that the failure of previous investigators to

note colorimetrically in uremic coma the marked increases in blood amino nitrogen which one of the writers (Kirk) has regularly observed by the gasometric method may have been due to the deficiency of the colorimetric method.   It also appears that absence of amino acidemia reported by the colorimetric method in other conditions cannot be accepted as conclusive.

Results with Urine

The *formaldehyde titration* and the *gasometric* method yielded results which agreed with each other as well as could be expected (Tables IV and V).   The fact that after gelatin feeding (Table V) the titration indicated somewhat greater increases of amino acid nitrogen excretion than the gasometric method, is presumably due to the exceptional richness of gelatin in proline and oxyproline. These two amino acids are determined in the formaldehyde titration but not in the gasometric method (Table I).   The formaldehyde titration and gasometric method both yielded accurate recoveries of amino acids added to urine.

With the *colorimetric* method, used as applied to urine (Folin, 1922, *b*), approximately accurate results were obtained with pure alanine solutions when standard and solution analyzed did not differ much (Table IV).   With the solution of mixed amino acids from casein, however, wide variations in results (from 9.2 to 28.6 mg. per cent) in the same solution were obtained when the size of the sample was varied, even though in no case did the difference between standard and unknown readings exceed a 1.5:1 ratio. The smaller the sample, the higher was the result obtained.   Urine showed a similar behavior.   When alanine was added to urine (bottom of Table IV) erratic results were obtained.

SUMMARY

The similarity of results obtained in urine with the gasometric method and the formaldehyde titration, and in blood by these two methods and the acetone titration, indicate that, when unusual interfering substances are absent, these methods serve as approximate measures of amino acid nitrogen.

The gasometric method and the formaldehyde titration appear to be generally applicable to both blood and urine, normal and abnormal.

The acetone titration has not yet been tried with urine. With blood it appears satisfactory unless there is an accumulation of organic acids. Lactic, acetoacetic, and other organic acids of similar strength require in the Zirm and Benedict application of the acetone titration 0.6 as much HCl per mol, and the weaker hydroxybutyric acid requires 0.14 as much, as do amino acids. Ordinarily the amounts of organic acids in the blood appear insufficient to cause serious errors. Accumulations of lactic acid, however, such as occur in severe exercise or anoxia, or of acetoacetic and hydroxybutyric acids such as occur in diabetic acidosis, would invalidate the acetone titration as a measure of the blood amino acids.

The colorimetric method of Folin, when applied to blood or urine in its original form, did not in our hands agree with the other methods, nor did it measure with accuracy amino acids added to either blood or urine.[4]

Of the two methods capable of general application to blood and urine, the gasometric method, in the micro form applied with the manometric apparatus, proved preferable for blood, with respect both to economy of material and convenience. In the urine, on the other hand, where economy of material is not necessary, and where the gasometric method involves removal of large amounts of urea, the Sørensen-Henriques formaldehyde titration in the form developed by Northrop proved the more convenient.

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[4] Danielson (1933), in a paper from Folin's laboratory which appears as this goes to press, publishes a modification of the colorimetric method which is stated to be less liable to error.

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THE EXISTENCE OF A UREA PRECURSOR DEPOT IN KIDNEY TISSUE

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Rehberg and Blem have recently (1932) found that when the urea contents of the fresh kidney tissues of frogs and rats are determined with both the urease and xanthydrol methods, higher values are yielded by the urease method.   As the most probable, although not definitely proved, explanation of this difference they presented the hypothesis that urea in the kidney is present in two forms, one which is diffusible and determinable both by the xanthydrol and urease methods, and another, "depot urea," which is not diffusible and is determinable by urease but not by xanthydrol. This latter modification they view as a precursor substance for some of the urea or ammonia of the urine.   Rehberg and Blem admit the possibility that the higher urea values obtained by the urease method might be due to the formation of ammonia by the action of arginase possibly present in the urease preparation on arginine present in the kidney tissue.   The reverse of this possibility, *viz.* the action of arginase or a similar tissue enzyme on a substrate in this crude urease, they do not appear to have considered.

The urease preparations from soy and jack beans used for urea determinations, however, are complex mixtures of proteins and other substances, and it appeared possible that the extra ammonia formation obtained by mixing the urease and tissue hash might arise from action of tissue enzyme on a substance in the urease preparation.   Such an explanation seemed the more probable, since Addis (1928) found that when liver hash was mixed with soy bean extract a large amount of ammonia was formed by action of an enzyme in the liver on a substrate in the bean extract.   Addis

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suggested that the substrate in the bean extract might be arginine or a derivative of it.   Recently, however, Kitagawa and Yamada (1932) have identified the substrate as another diamino acid, canavanin, which is hydrolyzed by liver extract to urea and canalin, according to the equation

$$C_6H_{12}O_3N_4 + H_2O = CO(NH_2)_2 + C_4H_{10}O_3N_2$$

Canavanin   Urea   Canalin
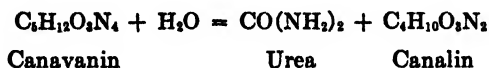
When the urease in the bean extract is not inactivated, the urea is at once converted into ammonium carbonate.

The experiments here presented have been carried out to ascertain whether, in urease-kidney mixtures, ammonia formed in excess of the urea present arises from action of urease on a substrate in the tissue, or from action of tissue enzyme on a substrate in the urease preparation.

<div style="text-align:center">EXPERIMENTAL</div>

The kidney tissue suspensions were prepared by grinding fresh kidney substance with sand and extracting the kidney hash with distilled water for 30 minutes.   Larger particles of connective tissue were removed by passing the suspension through a glass filter.

Urea determinations by means of urease were made by the aeration method of Van Slyke and Cullen (1916).   The urease preparation used (Squibb's) was prepared from jack bean extracts by acetone preparation as described by Van Slyke and Cullen (1914). 1 cc. of a 10 per cent aqueous urease solution was allowed to act for 1 hour at room temperature with 3 or 5 cc. of the kidney suspension, the mixture being buffered by addition of 5 cc. of a phosphate buffer solution, which contained 6 gm. of KH_2PO_4 and 2 gm. of Na_2HPO_4 per liter.

Xanthydrol determinations were made by the colorimetric method of Beattie (1928).   The proteins of the kidney suspension were precipitated by tungstic acid and 1 cc. of the filtrate, representing 0.1 cc. of kidney suspension, used for each determination. The accuracy of the colorimetric xanthydrol method was confirmed by comparing results obtained on tungstic acid blood filtrates with gasometric urease analyses of the same samples (see Table I).

Experiment 1, with Dog Kidney—54 gm. of kidney substance from a freshly killed dog were ground with sand and extracted with 100 cc. of distilled water at room temperature. The filtrate was made alkaline to litmus with potassium carbonate and concentrated *in vacuo* at 30–40° to remove preformed NH_3. After careful neutralization with acetic acid the solution was again made up to volume.

One portion of the kidney extract was boiled on a water bath for 10 minutes to destroy any enzymes present, and, after cooling, made up to the original volume.

Urea nitrogen determinations were made on boiled and unboiled samples of the kidney extract with both the aeration method of

TABLE I

Comparison of Urea Nitrogen Determinations on Tungstic Acid Blood Filtrates with Colorimetric Xanthydrol Method of Beattie and Gasometric Urease Method of Van Slyke

The figures represent urea nitrogen per 100 cc. of blood.

Xanthydrol	Gasometric urease
mg.	*mg.*
11 0	12.7
18.0	20.6
34.5	34.7
22.7	21.2
33.5	36.4
35 1	32.2

Van Slyke and Cullen and the colorimetric xanthydrol method of Beattie. The results are given in Table II. They show no significant differences between values yielded by the two methods with dog kidney tissue.

Experiment 2, with Dog Kidney—80 gm. of kidney substance from a freshly killed dog were ground with sand and extracted at room temperature with 200 cc. of distilled water for 30 minutes. One portion of the filtered extract was heated for 10 minutes on a water bath and, after cooling, made up to volume.

Urea determinations were performed as in the previous experiment with the urease aeration method and the xanthydrol method on boiled and unboiled samples. Preformed NH_3 was not removed,

but determined in separate samples by the aeration method. The results, in Table II, confirm those of Experiment 1.

Experiment 3, with Rat Kidney—The kidneys of five freshly killed rats, weighing 8.7 gm., were ground with sand and extracted with water for 30 minutes. One portion of the kidney extract was heated for 15 minutes in a boiling water bath, cooled, and made up

TABLE II

Urea Nitrogen per 100 Gm. of Fresh Kidney

Experiment No.	Source of sample	Unboiled samples		Boiled samples	
		Urease	Xanthydrol	Urease	Xanthydrol
		mg.	mg.	mg	mg.
1	Dog	31.7	34.4	27 9	29 7
		34.1	34.4	29.5	33 4
		32 0			
		35 1			
Average..............		33.2	34 4	28 7	31.5
2	Dog	69 2	70.7	69 3	71 5
		68 8		67.1	
		70.4		69.2	
Average..............		69 5	70.7	68 5	71 5
3	Rat	98 8	95.0	97.3	99 0
		98 9		98 9	
Average..............		98 9	95.0	98.1	99 0
4	Frog	7.4	0	0	0
		6.6	0	0	0
Average..............		7.0	0	0	0

to volume. Urea determinations were performed on unboiled and boiled samples by the aeration method of Van Slyke and Cullen and by the colorimetric xanthydrol method of Beattie. In the Van Slyke-Cullen determinations 90 minutes at 22° were allowed for the reaction of the urease solution. The results are given in Table II. They show no significant difference between values yielded by the two methods with rat kidney tissue.

Experiment 4, with Frog Kidney—The kidneys of six freshly killed bullfrogs (*Rana catesbiana*), of a total weight of 8.57 gm., were ground with sand, and extracted with 60 cc. of distilled water.

One portion of the filtered extract was heated in a water bath for 30 minutes (temperature of filtrate 93°), was afterwards cooled, and made up to volume. Preformed NH_3 was determined by the aeration method in boiled and unboiled samples, urea nitrogen in similar samples both by the aeration and xanthydrol methods. The urea and ammonia determinations were started 90 minutes after the death of the frogs. The results are given in Table II. They show distinctly higher urea values by urease determinations in unboiled samples of kidney extract than by xanthydrol determinations; whereas, no such difference was found in the samples where enzymatic activity of the extract had been destroyed by heating.

Test of Kidney Tissues for Arginase

For demonstration of the presence of arginase in fresh kidney tissue, arginine sulfate was added in solution to a sample of fresh kidney extract and to a similar sample in which enzymatic activity had been destroyed by heating in a boiling water bath. After allowance of sufficient time for any arginase present to act on the arginine, urea determinations were performed on both boiled and unboiled samples by the colorimetric xanthydrol method. Observation of higher urea values in the unboiled samples than in the boiled ones demonstrated the presence of arginase in the kidney tissue.

Test for Arginase in Kidney Tissue of Dogs—24.4 gm. of kidney substance from a freshly killed dog were ground with sand and extracted for 30 minutes with 100 cc. of distilled water. One portion of the extract was heated in a boiling water bath for 15 minutes, cooled, and made up to volume. To 5 cc. samples of boiled and unboiled extract were added 5 cc. of the same phosphate buffer solution used for the urea determinations, and 1 cc. of a 1 per cent solution of arginine (as arginine sulfate). The samples were left for 1 hour at 24°, and after this period made up to a volume of 15 cc. The diluted suspensions of boiled and unboiled extract were precipitated with tungstic acid, and urea determinations made in the filtrates by the xanthydrol method.

Test for Arginase in Kidney Tissue of Rats—The kidney extract was prepared as described under the experiment for comparison of urease and xanthydrol determinations in kidney tissue of rats. To 3 cc. samples of boiled and unboiled extract were added 5 cc. of phosphate buffer and 1 cc. of a 1 per cent solution of arginine

TABLE III

Test for Arginase in Kidney Tissue of Dog, Rat, and Bullfrog

Unboiled	Boiled
Urea nitrogen per 100 gm. of fresh kidney substance of dog after addition to kidney extract of an arginine solution	
mg.	*mg.*
99 0	89.5
89 5	89.5
Average . . 94 3	89 5 Result, no arginase present
Urea nitrogen per 100 gm. of fresh kidney substance of rat after addition to kidney extract of an arginine solution*	
131 0	129.2
128 7	
Average....129.4	129.2 Result, no arginase present
Urea nitrogen per 100 gm. of fresh kidney substance of bullfrog after addition to kidney extract of an arginine solution	
66.2	Faint trace
69.5	" "
Average.....67.9	Result, arginase present

* The arginine solution employed in this experiment was slightly decomposed (positive urea reaction with mercuric nitrate reagent). This accounts for the higher total values obtained in this than in the corresponding experiment on rat kidney.

(as arginine sulfate). The samples were left for 2 hours at 22°, and after this period made up to a volume of 10 cc. The diluted suspensions of boiled and unboiled extract were precipitated with tungstic acid, and urea determinations made in the filtrates by the xanthydrol method.

Test for Arginase in Kidney Tissue of Bullfrogs—7.25 gm. of kidney substance of freshly killed bullfrogs were ground with sand and extracted for 30 minutes with 40 cc. of distilled water. Arginine sulfate was added to boiled and unboiled samples of the kidney extract and the experiment performed as described above for demonstration of arginase in kidney tissue of the dog.

The results are given in Table III. They show no arginase activity of kidney tissue of the dog and rat, but a distinct activity of kidney tissue of bullfrog.

DISCUSSION

The ammonia formed from non-urea substances in mixtures of jack bean extract and frog kidney appears to be due, not as suggested by Rehberg and Blem to action of the jack bean urease on a urea precursor in the kidney tissue, but to interaction of an enzyme in the kidney tissue with material in the bean extract. Boiling the kidney tissue prevents the excess ammonia formation.

In mixtures of soy bean urease and liver, Addis (1928) has observed a similar formation of ammonia from tissue enzyme acting on a substrate in the urease preparation, and Kitagawa and Yamada (1932) have identified the substrate as a diamino acid, canavanin, similar to, but not identical with, arginine, from which arginase or a similar liver enzyme splits off quantitatively a molecule of urea. It appears probable that the same or a similar substrate is the source of the ammonia formation of non-urea origin noted by Rehberg and Blem. The ammonia formation noted in the mixture of bean extract and frog kidney does not appear to constitute evidence for the presence of a urea precursor in the kidneys.

CONCLUSIONS

The finding of Rehberg and Blem is confirmed, that in a mixture of jack bean extract (urease) and frog kidney more ammonia is formed than corresponds to the urea determined in the kidney tissue with xanthydrol.

The extra ammonia appears to arise, however, not from a substrate in the kidney tissue, but from a substrate in the bean extract, which is decomposed by arginase or a similar enzyme in the frog kidney.

In contrast to frog kidney, dog and rat kidney and jack bean

urease do not form ammonia from non-urea substances.   Likewise, frog kidney contains arginase, while dog kidney and rat kidney do not.

Even in frog kidney, the ammonia formation of non-urea origin appears to be evidence of an arginase-like enzyme in the tissue, and not of a urea precursor.

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STUDIES ON OXIDATION-REDUCTION*

XX. EPINEPHRINE AND RELATED COMPOUNDS†

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INTRODUCTION

Earlier papers in this series have been concerned largely with oxidation-reduction indicators. The value of such indicators as an aid to the determination of the generalized intensity factor of oxidation-reduction processes within living cells has been ably demonstrated by Cohen, Chambers, and Reznikoff (9). However, before chemical interpretations of the so called "oxidation-reduction potential" of living cells can be made with confidence there must be more definite knowledge of specific oxidation-reduction systems known to occur in living tissues. Among the small number of these systems which are amenable to direct potentiometric study are those in which certain, special derivatives of ortho- and paradihydroxybenzene are the reductants. The occurrence of these derivatives both in animal and plant cells is wide-spread. Some occur as the anomalous products of animal metabolism; others as hormones or poisons. In plants they have

TABLE I

Compounds Studied

Name	Donor or source	Description*
Benzohydroquinone	Eastman Kodak Company	M.p. 169.5°
Benzoquinone	" " "	Steam-distilled, m.p. 110–111°
Catechol	" " "	Original sample, m.p. 102–103°; 3 times recrystallized, m.p. 102–103°
Pyrogallol	" " "	
Gallic acid	" " "	
Gentisinic acid	Kahlbaum	Once recrystallized, m.p. 202–203°
Protocatechuic acid	"	Once recrystallized, m.p. 200°
Ethyl ester of gentisinic acid	Drs. L. Hellerman and M. E. Howard	M.p. 77.5–78°; prepared from Kahlbaum's gentisinic acid
Ethyl ester of protocatechuic acid	" "	M.p. 131.5–132°; prepared from Kahlbaum's protocatechuic acid
3,4-Dihydroxyphenylalanine	Hoffman-La Roche, Inc.	(1) Racemic, synthetic; (2) levo, from *Vicia faba;* once recrystallized, melting with decomposition at 275°
Epinephrine (bitartrate)	H. A. Metz Laboratories, Inc.	(1) Levo $[\alpha]_D^{25} = -51°$; (2) dextro $[\alpha]_D^{25} = +63°$. Both synthetic
Epinephrine (free base)	Parke, Davis and Company	Levo, from beef adrenals
" "	Dr. J. J. Abel	" " *Bufo agua*
Norhomoepinephrine	Dr. W. H. Hartung (Sharp and Dohme)	
Epinine	Dr. C. S. Leonard (Burroughs Wellcome and Company)	
Adrenalone	H. A. Metz Laboratories, Inc.	

* All melting points are uncorrected.

been associated with oxidative catalysis and with mechanisms of defense against infection. Some of them have been identified as intermediates in the formation of pigments.

It is of particular interest that the oxidants of practically all these compounds are unstable, especially in solutions having pH values within the physiological range and above. Therefore, if these compounds are to be protected from a virtually destructive oxidation in those natural environments which are reputed to be the seats of vigorous oxidative processes, the intensity level at which the general oxidative processes proceed must be below the level required for the oxidation of these specific substances. Accordingly data for the characteristic potentials of the systems of which these naturally occurring derivatives of catechol and benzohydroquinone are the reductants would help in defining the level of oxidation-reduction intensity of the living tissue in which they are found.

The systems which we have studied are indicated by the names of their respective reductants. These are listed with certain details in Table I. Included in this group are several synthetic compounds of pharmacological or structural interest.

Procedure

If an oxidation-reduction system have an unstable component, such changes may occur during the course of an ordinary titration as to make the interpretation of electrode potentials difficult or even impossible. Some success has been attained by quick titrations, as, for example, those of Clark, Cohen, and Gibbs (8) or by extrapolation methods such as were employed by Biilmann and Blom (3). Fieser (14) made discontinuous titrations with extrapolations of the time-potential curves and obtained satisfactory data for several unstable systems including that of catechol. However, these procedures were found to be inadequate for the purposes which are to be described. Accordingly Ball and Clark (2) adapted to electrode potential measurements the principle of rapid mixing and of observation of the flowing mixture; a procedure first employed for liquids by Hartridge and Roughton (17) and used by Schmidt (26), Saal (25), and Dirken and Mook (12) for the study of various aspects of ionic reaction.

The scarcity of certain of the substances precluded the use of

large volumes of solution and consequently made impracticable an apparatus of the type employed by previous workers. A diagram of the apparatus used, which was constructed entirely of glass, is shown in Fig. 1.
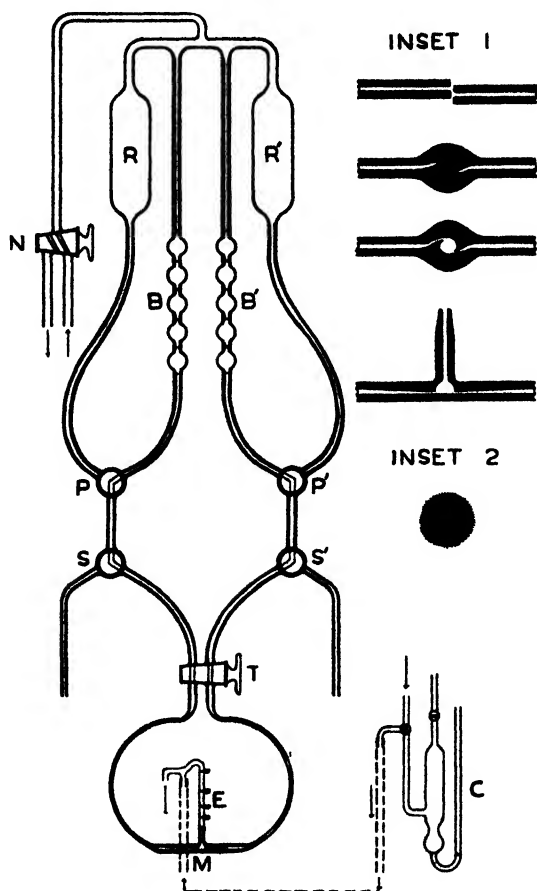
Fig. 1. Mixing apparatus and cell. R and R' are reservoirs; B, B', series of small bulbs; P, P', S, S', T, N, stop-cocks; M, mixing chamber; E, electrode chamber; C, calomel half-cell. Inset 1 shows construction of mixing chamber, M; Inset 2, platinum gauze sealed between sections of glass tubing. The diameter of the mixing chamber is 5 mm., depth 3 mm., inlets 0.15 mm., outlet 1 mm. The diameter of the electrode chamber is 3 to 4 mm., length 8 cm. The rate of flow of the mixture is approximately 20 ml. per minute.

Buffered solutions of the reductant (*e.g.* epinephrine) and an oxidizing agent (*e.g.* Ce(SO$_4$)$_2$) are stored in the 150 ml. reservoirs, R and R' respectively. Each of the series of five, small bulbs, B and B', can be filled from their respective reservoirs through the stop-cocks P, P'. The volume of each individual bulb is accurately calibrated; each holds about 2 ml. Before making a run, each set of bulbs is filled to the uppermost calibration mark and connected to the stop-cock T through the stop-cocks P, S and P', S' as shown. A stop-watch is started at the instant that stop-cock T is turned. There is then simultaneous flow of both solutions through the already filled system under a constant pressure (10 cm. of Hg) of purified nitrogen gas. This pressure, from a 12 liter reservoir, is applied through one arm of the stop-cock N. The two solutions meet at the mixing chamber, M, are thoroughly mixed, and pass to electrode chamber E attached by means of a ground glass joint. The mixture in passing through this chamber bathes each of a series of electrodes with a solution which is presumably of a fixed composition at any one electrode. After flowing past the last electrode, the mixture forms a flowing, liquid junction with a solution saturated with potassium chloride. The latter solution comes from a reservoir and is in direct connection with a saturated KCl calomel half-cell marked C. The potentials of the electrodes (usually four) and the time required for the emptying of any convenient number of bulbs of each set are measured. The ratio of oxidant to reductant at the moment of mixing can be calculated from the concentrations and the relative rates of flow of the two original solutions. This ratio can be varied by changing the concentrations of the solutions, or, as in the more usual case, by altering the setting of either stop-cock S or S'. Approximate adjustments of these settings are aided by pointers attached to the stop-cock keys. The time required for a given cross-section of the mixture to pass from the orifices in M to any electrode in E is calculated from the values for the rates of flow mentioned above and the calibrated space between the orifices in M and the given electrode. These data are employed in the extrapolation of the observed potentials to zero time and will be discussed more fully later.

The most important feature is the mixing chamber, the construction of which is illustrated by Inset 1, Fig. 1. A capillary

tube of Pyrex glass is broken and its ends placed in abutment but mutually offset.   During fusion of the junction in a flame, a slight pressure is exerted so that, as the walls of the capillary contract, their ends are turned away from one another.   A hole is then ground perpendicular to the capillaries and centered between their contracted ends.   This is cautiously widened until it makes contact with the ends of the capillaries.   The latter then open tangentially into this hole, which is the mixing chamber.   Both inlet orifices should have about the same diameter.   The interior of this chamber is ground smooth and then an outlet is fused on. This outlet carries the male end of a ground glass joint for connection to the female end of the electrode chamber.

The electrodes are of 80 mesh, platinum gauze sealed between sections of glass tubing (Inset 2, Fig. 1).   Contact is made by hooking platinum leads to platinum wire which has been wound around the protruding edges of each gauze electrode.   The electrodes are freshly plated with gold each time before use.   Goldplated electrodes come to equilibrium with the solutions more rapidly than blank platinum.

The whole apparatus is enclosed in a stirred, air thermostat. Most measurements were made at 30°.   All potentials here recorded have been brought to the hydrogen standard in accordance with the conventions of Clark (6).   In the tables, E'_0 will designate the potential of the system at 50 per cent reduction and a specified value of pH and E_0 the potential of the system at 50 per cent reduction extrapolated to pH $= 0$.

Corrections were made for change of potential attributable to those changes of pH which are caused by the products formed in the oxidation-reduction process.   Such corrections and minor manipulative procedures will not be described, since they involve numerous details and are similar to those described in previous papers of this series.   It was necessary to substitute veronal buffers as described by Michaelis (20) for the pH range covered by the borate system.   Borates cannot be used with polyhydroxy compounds.

Direct tests for completeness of mixture and reliability of potentials were made by applying the procedure to the comparatively stable system of which benzohydroquinone is the reductant.   Table II shows the results of one of several series of

such tests. Readings at all three electrodes agreed within 0.2 millivolt; the average is reported. The column headed "Running" gives the potentials observed with the mixture flowing past the electrodes, while that headed "Standing" gives the potentials which were observed after the flow had been stopped and the mixture allowed to stand in contact with the electrodes for several minutes. The agreement between the two sets of readings and between these readings and measurements taken in the ordinary way indicates that the mixing and reaction were practically com-

TABLE II

Hydroquinone (0.002 M) Versus Quinone (0.002 M)

pH 4.654; temperature $30.0° \pm 0.05°$; E'_0 (theoretical) $= 0.4158$. Assumed values, $E_0 = 0.6955$; $-\Delta E'_0/\Delta pH = 0.0601$.

Reduction	$0.03006 \log [S_r]/[S_o]$	E_h observed*		E'_0	
		Running	Standing	Running	Standing
per cent					
22 04	−0 0165	+0 4321	+0 4323	+0 4156	+0 4158
36 09	−0 0075	0 4233		0 4158	
43 35	−0 0035	0 4193	0 4192	0 4158	0 4157
55 09	+0 0027	0 4133	0 4133	0 4160	0 4160
55 25	0 0027	0 4132	0 4133	0 4159	0 4160
62 86	0 0069	0 4090	0 4093	0 4159	0 4162
71 35	0 0119	0 4040	0 4042	0 4159	0 4161
80 97	0 0189	0 3971	0 3974	0 4160	0 4163
Average..				0 4159	0 4160
By usual procedure				0 4158	

* Average of potentials at three electrodes.

plete before any given cross-section of the solution reached the first electrode and that there is no significant effect introduced by the streaming of the mixture past the electrodes. The largest deviation, 0.3 millivolt, is equivalent to a difference of about 0.5 per cent in the composition of the mixture. In general, deviations are small when the delivery from each jet is approximately the same. Variations in the rate of flow of the KCl solution had no detected effect. It is important to note that essentially similar results were obtained when, instead of mixing quinone and hydroquinone, hydroquinone was mixed with an oxidizing agent.

When we are dealing with a system, one component of which is unstable, we find the change of composition reflected in the change of potential from electrode to electrode.   Assume that the mixing is complete before a given cross-section reaches the first electrode and that, after mixing, the solution changes its composition with time in accordance with some law which is operating during mixing.   Then, if the rate of flow be uniform, the composition of the solution at any one electrode will be constant, even though the composition at one electrode differ from that at another electrode. Accordingly, and if the preliminary period of flow has been adequate, the lag in the adjustment of electrode potential to changing composition should be eliminated as it is not in the ordinary procedure of quick titrations.   Consequently if there can be found a uniform relation between the potentials at the several electrodes and the time at which a given cross-section of the flowing solution reaches each electrode, it should be possible to estimate by extrapolation the potential for the initial, undecomposed mixture.

In the majority of cases the observed relation was

$$-dE_h/dt = C \tag{1}$$

Equation 1 may be derived from simple assumptions.   Let the hydrion activity and ionic strength of a solution be constant. Then Equation 2 and its derivative, Equation 3, may be applied.

$$E_h = E_0' + \frac{RT}{nF} \ln \frac{[S_o]_t}{[S_r]_t} \tag{2}$$

$$\frac{dE_h}{dt} = \frac{RT}{nF} \frac{d[S_o]_t}{[S_o]_t dt} - \frac{RT}{nF} \frac{d[S_r]_t}{[S_r]_t dt} \tag{3}$$

$[S_o]_t$ and $[S_r]_t$ are the molar concentrations of total oxidant and total reductant at time t.   During mixing and reaction both $[S_o]$ and $[S_r]$ vary with time.   Let these processes be complete at one of the first and subsequent electrodes.   Then $[S_r]_t$ is a constant which may be calculated from the initial compositions of the unmixed solutions.   Also $d[S_r] = 0$.   Hence

$$\frac{dE_h}{dt} = \frac{RT}{nF} \frac{d[S_o]_t}{[S_o]_t dt} \tag{4}$$

Let the oxidant decompose in accordance with a pseudo first order reaction

$$\frac{-d[S_o]_t}{dt} = k[S_o]_t \tag{5}$$

Equations 4 and 5 give Equation 1. The experimental verification of Equation 1 is presumptive evidence of the validity of Equation 5. This has been found in the cases studied by Biilmann and Blom (3), by Phillips, Clark, and Cohen (23), and by Fieser (14). If the point giving the data for the first electrode falls upon the straight line passing through the other points, it is presumptive evidence that Equation 5 is applicable at the time of the first point and consequently that mixing and reaction had been practically complete before this time. Hence the potential corresponding to the ratio $[S_o]/[S_r]$, calculated as if there were no decomposition of oxidant, must be appreciably higher than that of the first electrode. If the line be extrapolated to zero time, the potential at the intercept would be that of a solution formed by instantaneous mixing and reaction. Since this cannot be the actual case, the potential at this intercept must be too high. Therefore the desired value should be somewhat higher than the potential at the first electrode and somewhat lower than the potential at the intercept.

Having no definite information about the course of the mixing and reaction, we have arbitrarily extrapolated to the zero time in each instance. Since the time for the first point was usually about 0.2 second and since there are given in Tables VI to X the values of $-\Delta E_h/\Delta t$ in millivolts per second, the reader may see that about one-fifth the value of $-\Delta E_h/\Delta t$ in any instance is somewhat *more than the maximum possible* error attributable to the theoretical difficulties discussed above.

In a complete analysis consideration should be given to the following. As an imagined cross-sectional surface of the mixture advances through the narrow electrode chamber, it should suffer distortion because of the friction at the walls. Hartridge and Roughton (17) have pointed out that this factor is minimized by the rotational motion imparted to the mixture as it is formed in the mixing chamber; but we have no information as to how far this type of flow will persist after passing a gauze electrode. There-

fore a gauze electrode placed perpendicular to the wall may not be exposed to a solution of strictly uniform composition.   The observed electrode potential will probably be that of the portion containing the largest percentage of undecomposed oxidant and this probably constitutes the greater part of the advancing section. However, since refined corrections to obtain precisely by extrapolation the true potential cannot be made, the matter under discussion needs no further consideration now.

TABLE III

Catechol (0.002 M) Oxidized with Ceric Sulfate (0.002 N).   In 0.1 M Acetate Buffer

pH 4.388; temperature 30.0° ± 0.05°.

Reduction	0 03006 log $[S_r]/[S_o]$	E_h^* observed	E'_0	E'_0† corrected
per cent				
15 14	−0.0225	+0.5520	+0 5295	+0 5289
22.99	−0 0158	0 5457	0 5299	0 5293
24.95	−0 0144	0 5443	0 5299	0 5293
39 38	−0 0056	0 5362	0 5306	0 5301
53 39	+0 0018	0 5281	0 5299	0 5294
59.77	0 0052	0 5248	0 5300	0 5295
59.99	0.0053	0 5251	0 5304	0 5299
74 99	0 0143	0 5167	0 5310	0 5306
80 83	0 0188	0 5116	0 5304	0 5300
86 18	0 0239	0 5064	0 5303	0.5300
Average......				0 5297
0.06011 × pH....				0.2637
E_0				0 7934

* Average of potentials at four electrodes.
† Corrected for acidity change due to oxidation-reduction process.

It would be desirable to extend the observations to include a time nearer that of the first contact between corresponding cross-sections of the initial solutions, since it might be possible, in certain instances, to obtain points on the chart closer to the potential axis at zero time.   However, several of our observations indicate that, while an improved apparatus might accomplish this, difficulties may be encountered in the inherent slowness of certain of the oxidation processes.   When unused oxidizing agent remains, there should be a competition between the two systems in their

effects upon the electrodes and this will seriously complicate interpretations. This competition seems evident in some of the results of Saal (25) who applied the principle of Hartridge and Roughton to the study of a variety of oxidation processes and who was able to make observations corresponding to 0.004 second after mixing.

In Tables III and IV are typical results testing the relation of Equation 2, where n has been regarded as 2. Although other systems with more unstable oxidants did not yield data agreeing

TABLE IV

Epinephrine (0.002 m) Oxidized with Ceric Sulfate (0.002 n). In 0.5 m Sulfuric Acid

pH $= 0.286$; temperature $30.0° \pm 0.05°$.

Reduction	$0.03006 \log [S_r]/[S_o]$	E_h^* observed	E'_0
per cent			
28 51	$-0\ 0119$	$+0\ 8030$	$+0\ 7911$
30.48	$-0\ 0108$	$0\ 8027$	$0\ 7919$
38.94	$-0\ 0059$	0.7969	$0\ 7910$
47 82	$-0\ 0011$	0.7916	$0\ 7905$
56.06	$+0\ 0032$	$0\ 7864$	0.7896
60.14	$0\ 0054$	$0\ 7850$	$0\ 7904$
81 88	$0\ 0197$	$0\ 7697$	$0\ 7894$
Average	.... . .	.. . .	$0\ 7906$
$0.06011 \times$ pH........	.. . .		$\underline{0.0172}$
E_0........	. ...		0.8078

* Average of potentials at four electrodes.

as well as do the data for catechol and epinephrine, there has been found no occasion to use any value for n other than 2.

All the cases studied should fall within Group A, Class 2 of the outline given by Clark and Cohen (7). The full equation, stated with numerical coefficient for 30°, is

$$E_h = E_0 + 0.03006 \log \frac{[S_o]}{[S_r]} + 0.03006 \log [K_{r_1}K_{r_2} + K_{r_1}(H^+) + (H^+)^2] \quad (6)$$

Here, in addition to symbols previously defined, (H^+) is the hydrion activity, and K_{r_1} and K_{r_2} are the apparent dissociation constants of those phenolic groups of the reductant which are

destroyed by the oxidation of the hydroquinone to the quinone. The dissociation constants must be defined as apparent because in the derivation of Equation 6 we have assumed constant ionic strength and solutions in which the buffer salts predominate.

Since several of the systems contain components having carboxyl or other acidic or basic groups not directly affected by the oxidation-reduction process but subject to change in strength by the operation of this process, the constants for these groups should doubtless be included in the equation. As noted by Clark and Cohen (7) and as illustrated in many subsequent experimental studies, such changes cause displacement of the curve relating E'_0 to pH. Some cases in which such displacements are suspected will be noted later. In no case do the experimental data permit accurate values to be assigned to the dissociation constants of these groups. Accordingly Equation 6 will suffice for the description of the results.

In most cases the instability of the oxidant increased so greatly as (H^+) decreased that it was impracticable to make measurements at values of (H^+) sufficiently low to make K_{r_1} and K_{r_2} significant in Equation 6. Hence most of the data are described by the simplified equation

$$E_h = E_0 + 0.03006 \log \frac{[S_o]}{[S_r]} - 0.0601 \text{ pH} \tag{7}$$

The case of catechol is peculiar and will receive special attention later.

Results

Table III contains the results of a typical experiment with catechol. The uniformity of the E'_0 values obtained over the range 15 to 86 per cent reduction is satisfactory. In solutions of the indicated pH the oxidant of catechol decomposes so rapidly that within 6 minutes after its formation nearly 50 per cent would have disappeared. Under such conditions the difficulty of obtaining a titration curve by the usual procedure can readily be appreciated. Yet in this experiment the rate of decomposition of the oxidant war relatively so slow that the potentials read at the four electrodes agreed within 0.1 millivolt and extrapolation was unneces-

sary. Such was also the case at all pH values less than 4.4[1] and not until the alkaline range was reached was extrapolation necessary.

An example of the results obtained with epinephrine is shown in Table IV. The oxidant of epinephrine is so unstable that it was only in the acid region that good agreement was obtained over a wide range of the titration curve. As pH increases, the rate of decomposition of the oxidant increases rapidly and it is necessary to resort to extrapolation of potentials in solutions with pH values greater than 2. In general, extrapolation of potentials was not employed when the value for $-\Delta E_h/\Delta t$ given in Tables VI to X was less than 0.1 millivolt per second. In Table V are the potentials obtained with several different preparations of epinephrine. Three of these are from widely different sources; yet no

TABLE V

Values for Several Preparations of Epinephrine Oxidized with Ceric Sulfate
Temperature 30.0° ± 0.05°.

Sample and origin	pH	E'_0	E_0
l-Synthetic; Metz	0 286	+0 791	+0 808
Beef glands; Parke, Davis	0 286	0 790	0 807
*Bufo aqua**.	0 286	0.791	0 808
l-Synthetic; Metz; $[\alpha]_D^{25} = -51°$	1 282	0 734	0 811
d-Synthetic; Metz $[\alpha]_D^{25} = +63°$	1 282	0.735	0.812

* A sample kindly supplied by Dr. Abel.

significant difference in potential was found. No significant difference in potential was observed for the systems of which *l*- and *d*-epinephrine are the reductants.

Available space will not permit the presentation of all the data in the detail shown in Tables III and IV. In the majority of cases the data fit the theoretical equation satisfactorily. Therefore the results may be summarized. Each value of E'_0 given in Tables VI to IX represents the average of at least four determinations at different percentages of reduction. In all cases a 0.002

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[1] Ceric sulfate can be used as an oxidizing agent at this pH only in acetate buffers. The usual rapid hydrolysis of ceric sulfate at this pH is retarded by the presence of the acetate ions; its oxidizing ability remains unaltered.

TABLE VI

Catechol and Epinephrine.   Relation of E'_0 and of $\Delta E_h/\Delta t$ to pH

Temperature 30.0°.

Oxidising agent	pH	Catechol			Epinephrine		
		E'_0	E_0 calculated assuming $\frac{-\Delta E'_0/}{\Delta pH} = 0.0601$	$-\dfrac{\Delta E_h}{\Delta t}$	E'_0	E_0 calculated assuming $\dfrac{-\Delta E'_0}{\Delta pH} = 0.0601$	$-\dfrac{\Delta E_h}{\Delta t}$
				mv. per sec.			*mv. per sec.*
Ce(SO$_4$)$_2$	0 286	0.772	0.789	0 033	0 791	0.808	0 006
"	0.906	0.737	0.791	0.010	0 755	0 809	0 02
"	1.280	0 714	0.791	0.011	0 733	0 809	0 03
Cl$_2$	1.390	0.707	0 791				
Ce(SO$_4$)$_2$	1.530	0.700	0.792	0 010	0 718	0.810	0 04
"	1.810	0 680	0.788	0.007	0.699	0 808	0.08
Br$_2$	2 680	0 628	0 789	0 005	0.645	0 806	0 42
Ce(SO$_4$)$_2$	3 237	0 594	0 788		0.609	0 804	2 0
Br$_2$	3 420				0 600	0 806	5 5
"	4.074	0.547	0 792	0.011	0 556	0 801	15 0
Ce(SO$_4$)$_2$	4 388	0 530	0.793	0.025			
Br$_2$	4 654	0 511	0.791	0.014			
Cl$_2$	4.654	0 514	0 791	0 018			
Br$_2$	4.815				0.515	0.804	25 0
Ce(SO$_4$)$_2$	5 031	0 489	0 792	0.027			
Br$_2$	5.241				0 497	0 812	90 0
"	6 079	0 427	0 793	0.040	0.430	0 796	30 0
Cl$_2$	6.083	0.428	0.793				
Br$_2$	6 828	0 385	0 795	0 16	0 390	0 800	30 0
K$_3$Fe(CN)$_6$	7.660	0 333	0 794	1.0	0 345	0.805	150 0
"	8.520	0.286		3.8			
"	8.549*	0.283		4.1			
"	9 276	0 253		9 0			
"	9.690*	0 239		17 5			
"	10 486*	0.198		29 2			
"	10.489	0.199		29 8			
"	10.910*	0.177					
"	11 291*	0 153		38 0			
"	11 661*	0.135		48.0			
"	12.068*	0.111		38.2			
"	12 528*	0.092					
"	12.997*	0.052		39.6			
Average..............			0.792			0.809 (first 5 values)	

* Catechol dissolved in conductivity water; oxidizing agent dissolved in buffer solution (see text).

M solution of the reductant and a 0.002 N solution of the oxidizing agent were employed.

Table VI contains a summary of the E'_0 values for both catechol and epinephrine. The E_0 values in both cases are calculated by assuming $-\Delta E'_0/\Delta pH = 0.0601$ at 30°. The average of the E_0 values obtained in the acid range places the normal potential of catechol at 0.792 volt for 30°. Ball and Clark (2) reported a normal potential of 0.798 volt for this system at 23°. The normal potential measured at 37.4° was lower than that at 30° by a corresponding amount. Fieser and Peters (15) reported the normal potential for this system at 25° to be 0.794 and 0.789 when the solvents were 0.5 N H_2SO_4 and 1.0 N H_2SO_4 respectively. These values are somewhat lower than those reported here when temperature differences are considered. The decrease in normal potential with increasing acidity noted by these workers is evident to a less degree in our results. We are inclined to attribute this effect to a change in the liquid junction potential with the more acid solution. We have found no occasion to employ a correction factor for an association between the oxidant and the reductant, as did Fieser and Peters. These workers do not state the concentration of catechol in their experiments, so no comparison is possible.

The highest pH value at which epinephrine could be studied with any satisfactory results was 7.66. Even here the potentials are not quite reliable. As indicated in Fig. 2, the values of E'_0 for the higher values of pH depart from the relation $-\Delta E'_0/\Delta pH = 0.0601$. This may be due to errors resulting from the great instability of the oxidant of the epinephrine system, although it might be due to a shift in the dissociation exponent of the substituted amino group incident to oxidation of the catechol group. Since the potentials observed with distinctly acid solutions are the more reliable, we may average these. On this basis the normal potential of the epinephrine system is 0.809 at 30.0°. The preliminary value reported by Ball and Clark (2) is undoubtedly too low.

Making due allowances for experimental errors, we find no indication that the value of the normal potential has been influenced by the type of oxidizing agent used. But it is interesting to note that chlorine acts much more slowly than does bromine.

Indeed, it was impracticable to use chlorine with epinephrine because the decomposition of the oxidant kept pace with the oxidation process.   The same was true of ceric sulfate in acetate buffer.   Such difficulties did not arise with catechol since its
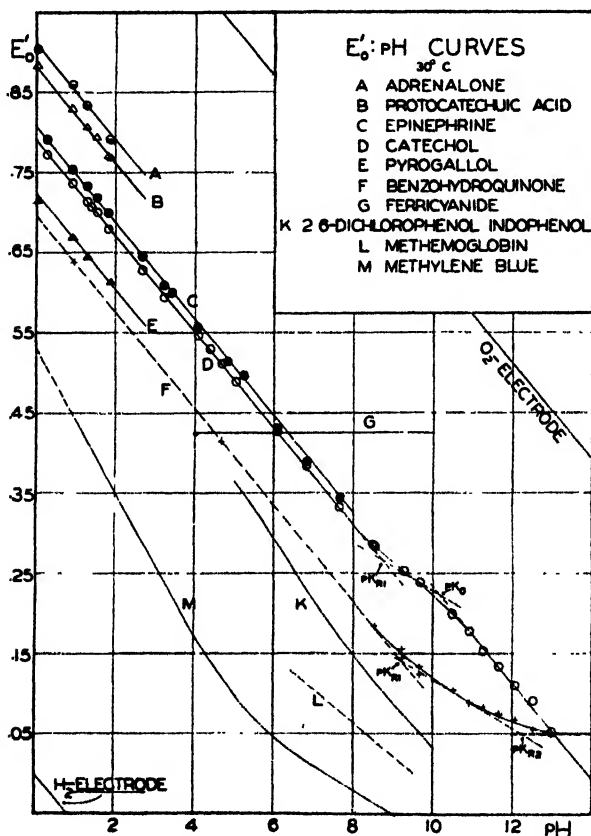
FIG. 2. Relation of E'_0 to pH

oxidant decomposed much more slowly than the oxidant of epinephrine.

The behavior of the catechol system at high pH is remarkable. As shown in Fig. 2, the curve relating E'_0 to pH undergoes two inflections.   As noted by Hall, Preisler, and Cohen (16), when the value of $-d^2E'_0/d\mathrm{pH}^2$ becomes negative, the dissociation causing

the change is assignable to the reductant; when the value becomes positive, the dissociation is that of the oxidant. Therefore the offset found here in the E'_0: pH curve, implying two inflections of opposite orientations, could be accounted for if we assume one dissociation exponent of catechol and, at a somewhat higher pH, one dissociation exponent of orthoquinone. This would demand of orthoquinone structural peculiarities. The dissociation of a hydrogen from the benzene nucleus or the addition of a hydroxyl ion might be imagined. Since the present data concern physical measurements, we shall proceed with the usual, *formal* assumptions which lead to Equation 8 containing constants defined as follows:

$$\frac{(H^+)\,[H\bar{R}ed.]}{[H_2Red.]} = K_{r_1} \qquad \frac{(II^+)\,[Re\bar{d}.]}{[HRed.^-]} = K_{r_2} \qquad \frac{(H^+)\,[Ox.^-]}{[HOx.]} = K_o$$

$$[S_r] = [Re\bar{d}.] + [H\bar{R}ed.] + [H_2Red.]$$

$$[S_o] = [HOx.] + [O\dot{x}.]$$

In deriving Equation 8, it is assumed that the second dissociation constant of catechol is negligibly small.

$$E_h = E_0 + 0.03006 \log \frac{[S_o]}{[S_r]} + 0.03006 \log \frac{(H^+)^3 + K_{r_1}\,(H^+)^2}{K_o + (H^+)} \qquad (8)$$

The curve of Fig. 2 is drawn to correspond with this equation when $E_0 = 0.792$, $K_{r_1} = 1.79 \times 10^{-9}$, and $K_o = 6.31 \times 10^{-11}$.

It is recognized that the experimental data may be at fault, since, in the region of pH where this inflection of the curve occurs, the oxidant is very unstable. However, the data obtained at any one pH value showed good agreement among values of E'_0 calculated for different percentages of reduction. In order to obtain a check on the validity of the data, we have made a similar study of the system benzohydroquinone-benzoquinone in this same pH range. The data are presented in Fig. 2, Curve F. The curve drawn to fit the data is expressed by Equation 6 in which $E_0 = 0.6955$, $K_{r_1} = 6.61 \times 10^{-10}$, and $K_{r_2} = 5.25 \times 10^{-12}$. The results obtained are in complete accord with the expected behavior of this system and give no evidence that the unstable paraquinone behaves as the orthoquinone does.

In the majority of cases the data presented for the catechol system in the alkaline range were obtained by running a solution of the reductant in conductivity water against a buffered solution of ferricyanide.   In the case of the benzohydroquinone system this same procedure was followed and in addition each point was checked by running an equimolecular mixture of quinone and hydroquinone in conductivity water against a plain buffer solution. Both these procedures were found to give the values obtained by

TABLE VII

l-3,4-Dihydroxyphenylalanine.   Relation of E'_0 and of $-\Delta E_h/\Delta t$ to pH.
Temperature 30.0°.

Oxidizing agent	pH	E'_0	E_0 calculated assuming $-\Delta E'_0/\Delta pH = 0.0601$	$-\Delta E_h/\Delta t$
				mv per sec.
Ce(SO₄)₂	0 028	0 802	0 804	0 08
"	0 919	0 753	0 808	0 15
"	1 295	0 726	0 8C4	0 28
"	1 295	0 725*	0.803	0 27
"	1 832	0 686	0 797	0 23
"	1 832	0 688†	0 798	0.30
"	1 887	0 685	0 798	0 33
Br₂	2 670	0 635	0 795	1 3
"	3 398	0.589	0 793	2 2
Ce(SO₄)₂	4 130	0.543	0 791	0 28
"	4 694	0 511	0 793	0 81
Br₂	6 076	0 435	0 798	
"	6 702	0 393	0 796	35 0
Fe(CN)₆ᵐ	7 664	0 326	0 787	64.0

* Synthetic racemic sample.
† Unrecrystallized sample.

the employment of the usual technique described earlier.   Changes in pH due to dilution of the buffer were determined and the potentials were corrected accordingly.

   Table VII shows the relation of E'_0 and of $-\Delta E_h/\Delta t$ to pH for the system whose reductant is the amino acid, 3,4-dihydroxyphenylalanine.   One run was made with a synthetic *dl* preparation at pH 1.295.   The value obtained agreed well with that given by the levo preparation at the same pH.   This is in agreement

TABLE VIII

Relation of E'_0 and of $-\Delta E_h/\Delta t$ to pH

Temperature 30.0°.

pH	Protocatechuic acid			Ethyl ester of protocatechuic acid			Gentisinic acid			Ethyl ester of gentisinic acid			Pyrogallol			Gallic acid		
	E'_0	E_0*	$-\dfrac{\Delta E_h}{\Delta t}$	E'_0	E_0*	$-\dfrac{\Delta E_h}{\Delta t}$	E'_0	E_0*	$-\dfrac{\Delta E_h}{\Delta t}$	E'_0	E_0*	$-\dfrac{\Delta E_h}{\Delta t}$	E'_0	E_0*	$-\dfrac{\Delta E_h}{\Delta t}$	E'_0	E_0*	$-\dfrac{\Delta E_h}{\Delta t}$
			mv. per sec.			mv. per sec.			mv. per sec.			mv. per sec.			mv. per sec.			mv. per sec.
0.028	0.883	0 884	0 10	0 883	0 885	0.03	0 795	0 796	4.1	0.792	0 794	0 09	0.715	0.716	60	0.807	0.809	66
0.919	0.829	0 884	0.09	0.830	0 885	0 03	0.740	0 795	2 2	0 738	0.793	0.08	0.652	0.707	26	0.742	0.797	33
1.289	0 806	0.884	0 07	0.807	0 885	0 03							0 638	0.715	37	0.724	0 801	37
1.295							0 714	0 792	8 3	0 715	0 793	0 15						
1.522	0.794	0.886	0 14	0 792	0 883	0 03												
1.832	0.770	0.880	0.11															
1.887	0.768	0.881	0.11	0.771	0.884	0 03	0 674	0.788	2.8	0.680	0 793	0 20	0 603	0.716	25	0.674	0.788	41
Average..		0.883			0.884			0 793			0.793			0.713			0.799	

* Calculated, assuming $-\Delta E'_0/\Delta pH = 0.0601$.

with the results obtained with the isomers of epinephrine. The values for $-\Delta E'_0/\Delta pH$ are fairly close to 0.0601 at pH values greater than 2. At lower pH values there is a deviation in the direction of a "0.09 slope." This can be seen from the increase in the calculated E_0 values in this pH range. It is to be expected that within this pH range a shift in the E'_0: pH curve could occur due to the difference in the dissociation constants of an ionizable group, either carboxyl or amino, of the reductant and the oxidant. The shift towards a "0.09 slope" indicates that the ionizable group of the oxidant is stronger than that of the reductant. Blix (4)

TABLE IX

Relation of E'_0 and of $-\Delta E_h/\Delta t$ to pH for Several Pressor Substances
Temperature 30.0°.

pH	Adrenalone			Epinine			Norhomoepinephrine		
	E'_0	E_0*	$-\dfrac{\Delta E_h}{\Delta t}$	E'_0	E_0*	$-\dfrac{\Delta E_h}{\Delta t}$	E'_0	E_0*	$-\dfrac{\Delta E_h}{\Delta t}$
			mv. per sec.			*mv. per sec.*			*mv. per sec.*
0.028	0 905	0 906	4 1	0 783	0 785	0 012	0 817	0 818	0 015
0.919	0.860	0.915	6 0	0 735	0 790	0 002	0 769	0 825	0 01
1.289				0 712	0 790	0 007	0 745	0.823	0 004
1.295	0.833	0.911	2 8						
1.887	0.791	0 905	7 4	0 676	0.789	0 013	0 708	0 821	
4.130				0.539	0.788	2 0			
7.664				0 306	0.767†	168 0			
Average.....		0 909			0 788			0 822	

* Calculated, assuming $-\Delta E'_0/\Delta pH = 0.0601$.
† Value excluded from the average.

found the carboxyl group of the oxidant of the homogentisic acid system to be more easily ionized than the same group in the reductant. The first obvious change in the E'_0: pH curve for dihydroxyphenylalanine occurs between pH 1 and 2. This is in agreement with the pK′ value, 2.26, assigned by Miyamoto and Schmidt (21) to one of the ionizable groups of this amino acid. The data obtained in this study are not accurate enough to enable us to assign an exact value to the dissociation constant.

In Table VIII are assembled data showing the relation of E'_0 and of $-\Delta E_h/\Delta t$ to pH for six compounds. With the exception

of the ethyl esters of protocatechuic and gentisinic acids all of these compounds are known to occur in plants. The slight decrease in the E_0 values at pH 1.887 for the compounds with a carboxyl group attached to the ring may be due to the effect of the difference in dissociation constants for this group in the reductant and oxidant as discussed in the case of dihydroxyphenylalanine. The fact that the ethyl esters show no such change substantiates this supposition.

Table IX contains values showing the relation of E'_0 and of $-\Delta E_h/\Delta t$ to pH for three, synthetic, pressor substances.

The normal potentials for all systems studied are summarized in Table X.

DISCUSSION

It is of interest to observe the relation between structure and free energy change. A comparison of the data on benzohydroquinone and catechol shows that catechol has a normal potential nearly 100 millivolts more positive than that of benzohydroquinone. The difference in normal potential between protocatechuic acid and gentisinic acid is of the same order of magnitude. This characteristic potential difference between the para and the ortho isomers is not confined to the quinones; Conant and Pratt (11) have shown the same for quinoimines. The normal potentials of epinephrine, 3,4-dihydroxyphenylalanine, and epinine are all very close to that of catechol. The various side chains apparently do not modify greatly the normal potential of catechol. These experimental facts are in agreement with the usual rule that compounds of similar chemical structures possess approximately the same normal potential. As an exception, however, norhomoepinephrine has a normal potential 30 millivolts more positive than that of catechol. Pyrogallol is a "stronger" reducing agent than catechol by reason of the substitution of a hydroxyl group. A difference of 79 millivolts exists between the two systems. Conant and Fieser (10) and Fieser (13) have reported similar results for the substitution of a hydroxyl group in other systems. These same workers have also shown that the substitution of a carboxyl group raises the normal potential. Among the compounds studied in the present investigation, the following three pairs, pyrogallol-gallic acid, catechol-protocatechuic acid, and

benzohydroquinone-gentisinic acid, show the effect of such a substitution.   The effect of the carboxyl group is to raise the normal potential 86, 91, and 97 millivolts respectively.   The potential of

TABLE X

Summary of E_0 and $- \Delta E_h/\Delta t$ for All Compounds
Temperature 30.0°.

Reductant	E_0	$- \dfrac{\Delta E_h}{\Delta t}$ at pH 1.3
HO HO—◇, catechol..............	0.792	0.011
R—CHOH·CH₂·NH·CH₃, epinephrine.............	0 809	0.03
R—CH₂·CH·NH₂·COOH, dihydroxyphenylalanine.	0.800	0 28
R—CH₂·CH₂·NH·CH₃, epinine........	0.788	0.007
R—CHOH·CH·NH₂·CH₃, norhomoepinephrine.....	0 822	0.004
R—CO·CH₂·NH·CH₃, adrenalone	0.909	2.8
R—COOH, protocatechuic acid	0 883	0.07
R—COOC₂H₅, ethyl ester of protocatechuic acid..	0 884	0.03
HO HO—◇, pyrogallol........... .... HO	0.713	37.0
HO HO—◇COOH, gallic acid..................... HO	0 799	37.0
HO—◇OH, gentisinic acid.................... COOH	0.793	8.3
HO—◇OH, ethyl ester of gentisinic acid ...... COOC₂H₅	0 793	0.15
HO—◇OH, benzohydroquinone.............. .	0.696	

R = 3,4-dihydroxyphenyl.

adrenalone was found to be 118 millivolts higher than that of catechol, presumably because of the carbonyl group attached to the benzene ring.   It appears that the effect of substitution of one carboxyl group is to produce an increase of normal potential of

the same order of magnitude, provided the parent compounds are comparable in other respects.

An idea of the rapidity with which the oxidant of any compound decomposes can be obtained from the data listed in Tables VI to X under the column "$-\Delta E_h/\Delta t$;" i.e., the change of potential in millivolts per second. These data were obtained, either by following the drift of potential with time after stopping the flow of the mixture, or directly from the potentials at the various electrodes during active flow of the mixture. Changes in the initial concentration of oxidant at fixed pH caused but little change in the value of $-\Delta E_h/\Delta t$ when the percentage of oxidation was 50 per cent or less. At higher percentages of oxidation the value of $-\Delta E_h/\Delta t$ tended to increase in some instances, though the relationship remained linear. Values reported are for an approximately equimolecular mixture of oxidant and reductant. A change of potential of about 9 millivolts corresponds to the disappearance of half the amount of oxidant originally present in an equimolecular mixture. Hence the so called "half life" of an oxidant, in seconds, can be readily obtained by dividing 9 millivolts by the given value of $-\Delta E_h/\Delta t$. For example, the value of $-\Delta E_h/\Delta t$ for epinephrine at pH 7.66 and 30° is 150 millivolts per second. Therefore the "half life" of the oxidant is 0.06 second. The velocity constant, k, for the decomposition of any oxidant can be calculated from the following equation obtained by combining Equations 4 and 5. Since $-\Delta E_h/\Delta t$ is expressed in Tables VI to X as millivolts per second, instead of volts per second, the factor 1000 is used in Equation 9.

$$k = nF/1000\, RT\ (-\Delta E_h/\Delta t) \tag{9}$$

In the case of epinephrine, cited above, $k = 11.5$.

The orthoquinones are usually more unstable than the paraquinones. As an exception, the oxidant of gentisinic acid is more unstable than the oxidant of protocatechuic acid. In general the substitution of an ionizable side chain increases the inherent instability of a quinone as noted by Nef (22), who found it difficult to prepare the oxidants of several hydroquinone carboxylic acids. This effect is seen on comparing the results for systems in which an acid and its ethyl ester respectively are the reductants. Although there is but an insignificant difference in the normal

potentials, there is a marked difference in the stabilities of the oxidants.

Attempts were made to develop *quantitative* relations between the observed values of $-\Delta E'_0/\Delta t$ and pH.   No simple relation was discovered.   Qualitatively it may be said that, with the exception of some anomalous effects in very acid solution, the instability of each oxidant increases rapidly with increase of the pH value of the solution.   Also at 37.4° the oxidant of catechol is about twice and that of epinephrine about thrice as unstable as at 30°.

On p. 705 are mentioned the normal potentials of the catechol system at different temperatures.   A comparable fall of potential with increase of temperature was noted for the epinephrine system. We have not attempted accurate estimates of the temperature coefficients of the normal potentials of the several systems because there are lacking certain basic data for corrections to an acceptable basis.   However, neglect of accurate temperature coefficients will not be significant in the following discussion.

The naturally occurring derivatives of catechol and of benzo-hydroquinone are reductants of systems having characteristic potentials which are distinctly positive to that of the system of which 2,6-dichlorophenol indophenol is the oxidant.   This dye is rapidly and completely reduced by most living cells.   Since there is no reason to believe that either this dye or any one of the quinones requires specific reducing agents or specially catalyzed reducing processes, the free energy data seem sufficient for the following conclusion.   Cells having systems capable of completely reducing 2,6-dichlorophenol indophenol must be capable of maintaining the systems now under discussion extensively in the reduced state.   The argument can be elaborated by consideration of the conduct in cells or tissues of other indicators or of other naturally occurring systems such as the hemoglobin-methemo-globin system.

Conversely, the fact that several of the reductants of the systems now under consideration are found in cells or in their immediate environment, and at levels of pH which would ordinarily enhance the ease of their oxidation by oxygen, may be used in support of the impression, gained from various sources, that the level of the generalized intensity factor of the free energy of cellular oxidation and reduction is well below that required to maintain in a tem-

porary state of equilibrium any appreciable amount of the quinones in question. This conclusion is strengthened when we consider the apparent protective action of cells toward the reductants in question in relation to the peculiarities arising from the inherent instability of the oxidants. The significance of the latter fact can best be shown by noting the errors which have arisen in efforts to evaluate the oxidation-reduction potentials of the epinephrine and related systems by their behavior toward dyes of known characteristics. Kendall (18) reported that 2,6-dibromophenol indophenol was reduced by epinephrine and by adrenalone at pH 7.4. Blix (4) reported this same dye, as well as o-cresol indophenol, to be partly reduced by epinephrine at pH 7.0. However, at pH 7.0, for example, equivalent amounts of epinephrine and 2,6-dichlorophenol indophenol[2] would react so that *initially* about 0.1 per cent of the dye would be reduced and the equivalent amount of the epinephrine would be oxidized. The oxidized epinephrine would quickly decompose and to maintain the flow of energy further reaction would occur. By the constant withdrawal of the oxidized epinephrine through decomposition the reaction would go to completion with complete reduction of the dye. Neglect of the decomposition and use of the ordinary interpretation of indicator conduct leads to the false conclusion that the potential of the epinephrine system is negative to that of the dye systems; whereas, in fact, the opposite is true. We have found that when equivalent amounts of epinephrine and 2,6-dichlorophenol indophenol are dissolved in a buffer solution of pH 7.0 and in the absence of oxygen, the dye is completely decolorized within about 1 hour. The red decomposition product usually obtained upon oxidizing epinephrine does not appear. Potentials measured during the course of the reaction progressively decline, lie within the region characteristic of the dye, and are not those to be expected if the dye system alone were influencing the electrode. It is possible to restore the original color of the dye to practically full intensity by the addition of an equivalent amount of ferricyanide. The dye is then slowly decolorized again. This may be repeated. In short, 1 equivalent of epinephrine is capable of decolorizing 4 equivalents of the dye. If epinephrine be first

[2] The potentials characteristic of the dichloro- and dibromoindophenols are nearly the same.

completely oxidized by ferricyanide with the formation of the usual red product and then dye be added in amount equivalent to the initial epinephrine, decolorization takes place and the same number of equivalents are used as in the experiment previously mentioned.   These facts indicate that decolorization of the dye is due to reduction, although the absence of the red decomposition product of oxidized epinephrine, the peculiar conduct of electrodes, and the reduction of 4 equivalents of dye suggest side reactions. Several types of these might be postulated from the known conduct of analogous compounds.   Undoubtedly there are concerned processes similar to those outlined by Raper (24) in his report upon the oxidation of dihydroxyphenylalanine to melanin.

Chambers, Cohen, and Pollack (5) have noted that the use of an oxidation-reduction indicator system, the reductant of which is unstable (as is one reductant of Janus green), is subject to the serious objection that such an indicator may be completely re-duced by another more electropositive system.   We now suggest that care be used in the interpretation of results obtained upon applying *stable* indicator systems to systems with unstable com-ponents.   Since many biologically important oxidation-reduction systems are of this category, there will arise the question whether the reduction of a dye by a cell is indicative of a level of oxidation-reduction intensity pertaining to a true equilibrium state.   Re-sults with such situations should be scrutinized with especial care when increasingly more negative indicator systems require longer times for reduction, since such a set of relations is what may be expected if the dye is being reduced by a more positive system with unstable oxidant.   Of this pattern are most of the results of the microinjection studies.

It is interesting to note that the systems of which *l*- and *d*-epinephrine are the reductants show no significant difference in potential.   It is to be expected that the free energies of optical isomers will be identical, since they are produced in equal quan-tities when synthesized *in vitro*.   While the precision of the present investigation is inadequate to give this proposition a severe test, the results are not inconsistent with the prediction and constitute what we believe to be the first recorded test.   These results also show that the pharmacological action of a compound is not de-pendent on the free energy level of its system since this level is the same in both the cases under consideration, while the difference in

pharmacological action is pronounced. This statement must not be construed to imply that the oxidation-reduction intensity of the environment will not affect the pharmacological action of a compound.

It is well known that the intravenous injection of epinephrine into an animal causes a pronounced rise of blood pressure which is, however, of short duration. Can this transient action be connected in any way with the oxidation and consequent destruction of this hormone at the site of its action? The fact that ephedrine, which contains no orthohydroxy groups and so should be less easily oxidized and destroyed, has a prolonged effect on blood pressure is suggestive, though it must be remembered that this difference may be due to a difference in the site of action. Such a mechanism, however, would demand either that the tissues where epinephrine is synthesized or stored have a reducing intensity widely different from those tissues on which it exerts its action, or else epinephrine is liberated from a precursor at the site of action. The existence of epinephrine precursors in the body has been reported by Kendall (19) and by Annau, St. Huszák, Svirbely, and Szent-Györgyi (1).

The mechanism of the oxidase of the higher plants has been assumed to be the formation of an organic peroxide from the dihydroxy group characteristic of catechol. Szent-Györgyi (27) has suggested that an orthoquinone is formed. If any of the compounds studied here are to act even at low pH values as cyclic catalysts by the formation of orthoquinones, either the process must be a comparatively rapid one, or else some mechanism to stabilize the orthoquinone is involved; otherwise an appreciable destruction of the catalyst would occur.

The oxidation of many of these compounds results in the formation of colored products. These colored products are not the quinones that are first formed. This is apparent from the fact that color intensity is still at a maximum when electrode potentials indicate that all quinone has disappeared. This has a bearing upon the usual colorimetric methods of analysis.

SUMMARY

An apparatus for the study of the oxidation-reduction potentials of unstable systems is described. With this apparatus thirteen systems, the stable reductants of which are catechol, benzohydro-

quinone, or their derivatives, were studied at 30° and various pH values.   The curve relating pH and the potentials of the half reduced solutions, E'_0, for each system has a slope $-\Delta E'_0/\Delta pH = 0.06$ in acid solution.   The instability of the oxidants of most of these systems increased to such an extent in the alkaline range that only two, catechol and hydroquinone, were amenable to study in solutions with pH values greater than 8.0   The data obtained for the benzohydroquinone system were in accordance with theoretical expectations, 2 hydrions dissociating from the reductant as pH increased so that $-\Delta E'_0/\Delta pH$ approached zero. The dissociation constants were assigned the values $K_{r_1} = 6.61 \times 10^{-10}$ and $K_{r_2} = 5.25 \times 10^{-13}$.   The catechol system was peculiar in that the data for the alkaline range required the assumption of a dissociable hydrion of the oxidant and the dissociation of only 1 hydrion from the reductant.   The values for these dissociation constants are $K_{r_1} = 1.78 \times 10^{-9}$ and $K_o = 6.31 \times 10^{-11}$.

The relation of structure and free energy is discussed.   The results are in general agreement with those found for other systems. The systems of which the isomers of epinephrine are the reductants are found to possess the same normal potential.

The change of potential with time is used as the criterion of the instability of an oxidant.   In general the data show that the substitution of an ionizable side chain markedly increases the inherent instability of quinones and that the oxidants of all systems become more unstable as pH increases.

The potentials of the naturally occurring systems were found to be so positive that living tissues under normal physiological conditions can keep them practically completely in the reduced state.   The reductants of these systems are thereby protected from a virtually destructive oxidation.

The reduction of an oxidation-reduction indicator by a more positive but unstable system is noted.   It is emphasized that results obtained by means of oxidation-reduction indicators on unstable systems or on biological material where such systems are present must be interpreted with great care.

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THE FATE OF MANNITOL AND MANNITAN IN THE ANIMAL BODY

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A few years ago two of the authors (C. J. C. and J. C. K. (1)) became interested in the metabolism of the inulin obtained from burdock root. The hydrolysis of this polysaccharide, with the formation of levulose, directed their attention to the secondary alcohol, mannitol, and its first anhydride, mannitan.

The dried saccharin exudation, manna, obtained from *Fraxinus ornus* has been used for years in the medicine of folk-lore as a laxative. In 1883 Jaffe (2) observed that mannitol could be fed to dogs and recovered unchanged in large quantities from the urine. In rabbits the compound was partially decomposed. The resistance to oxidation in the body of mannitol is indeed interesting when contrasted with the behavior of sorbitol (3) which readily undergoes oxidation.

Sollmann (4) suggests the use of mannitol as a sweetening agent in the diabetic diet. In 1919 Field (5) studied the effect of the ingestion of 100 gm. of mannitol and certain other compounds on the blood sugar curves of normal colored males. The maximum rise with glucose was 40 mg. per 100 cc.; with mannitol the maximum rise was 10 mg. per 100 cc. The failure of mannitol to relieve insulin shock in white rats was observed by Voegtlin *et al.* (6). In 1929 Ariyama and Takahashi (7) studied the growth curves of white rats on an adequate diet with various carbohydrates. Mannitol was found to be far inferior to glycerol or ethyl alcohol. The bacteriological decomposition of mannitol has been investi-

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gated by many workers.   This work is reviewed comprehensively by Eitel (8).

The authors found no account of the metabolism of mannitan in the literature.   The purpose of this investigation is to study the fate of mannitol and its first anhydride, mannitan, in the animal body.

Materials Employed—The mannitol used in this investigation was supplied through the courtesy of Merck and Company, Inc., Rahway, New Jersey.   The aqueous solution of the compound (1:10) is neutral to litmus and optically active.   The mannitan was prepared from mannitol by a slight modification of the method of Vignon (9) in which the dehydration is accomplished by means of sulfuric acid.   The compound was acetylated and the resulting ester distilled *in vacuo*.   The fraction distilling between 200–210° at 10 mm. was saponified.   The calculated percentage of CH_3COOH is 72.31, that found was 71.70.   Through the chemical investigations of van Romburgh and van der Burg (10) who showed that α-vinyldihydrofurane could be obtained from mannitan tetraformate by reduction with hydrogen, the following structure has been assigned to mannitan and its relationship to mannitol may be observed.

Mannitol	Mannitan

$$
\begin{array}{cc}
\text{Mannitol} & \text{Mannitan} \\
\mathrm{CH_2 \cdot OH} & \mathrm{CH_2 \cdot OH} \\
| & | \\
\mathrm{H-C-OH} & \mathrm{H-C-OH} \\
| & | \\
\mathrm{H-C-OH} & \mathrm{H-C} \\
| & | \\
\mathrm{HO-C-H} & \mathrm{HO-C-H} \quad O \\
| & | \\
\mathrm{HO-C-H} & \mathrm{HO-C-H} \\
| & | \\
\mathrm{CH_2 \cdot OH} & \mathrm{H_2C}
\end{array}
$$
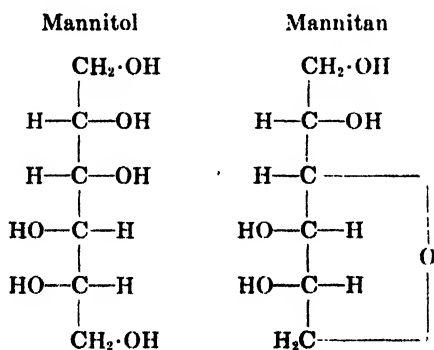
Glycogen Storage with Mannitol and Mannitan in Livers of White Rats—White rats were fasted for a period of 24 hours and then placed in small individual cages.   The control rats were given a weighed liberal supply of cacao-butter and allowed to continue on this diet of fat for a period of 80 hours.   The experimental rats were fed mixtures of cacao-butter and mannitol and

mannitan respectively. The mixtures contained 33 per cent of mannitol and mannitan. The rats were then killed by exsanguination; the livers were immediately extirpated and the glycogen estimated by Pflüger's (11) method. The glucose formed by the hydrolysis of the glycogen was determined by the Shaffer and Hartmann (12) modification of the Folin-Wu method. In certain instances the rats were grouped and their livers analyzed. A few experiments were conducted with levulose for the purposes of comparison. The glycogen content of four livers was 2.89 per cent. The results of these experiments are shown in Table I.

A summary of Table I shows that the rats fed on a basal fat diet ingested an average of 10.9 gm. of fat; whereas, those fed the experimental diet with mannitol ingested an average of 9.3 gm. of food, 67 per cent of which was fat. Hence the amount of fat ingested by these rats was actually less than that ingested by the control rats; therefore, it is concluded that the additional glycogen storage is due to the presence of mannitol in the diet. On the other hand, mannitan does not give rise to increased glycogen storage, and in more than 70 per cent of the experiments the glycogen content of the liver was less than an amount capable of being detected by analytical procedure. By the method it is possible to detect 0.01 per cent of glycogen when a charge of 5 gm. of tissue is used. In an effort to establish the fate of these compounds in the body a series of experiments was conducted to measure the gaseous exchange of the white rat after the ingestion of each substance.

Effect of Mannitol and Mannitan on Respiratory Quotient— With certain minor modifications, Haldane's (14) open circuit apparatus was employed to measure the respiratory quotients.

The animals used in these experiments were fed a ration consisting of cod liver oil, wheat meal, corn-meal, lard, and meat scrapple. They were fasted for 48 hours prior to the experiment. No correction was made for the nitrogen content of the urine of the animals. This was deemed unnecessary for the purpose of this investigation as Cori and Cori (15) have shown that approximately 90 per cent of the metabolism in a fasting rat is fat oxidation.

The results of respiratory determinations on eight fasting rats for $2\frac{1}{2}$ hour periods were 0.708, 0.703, 0.701, 0.709, 0.701, 0.690,

0.702, and 0.718 with a mean of 0.704.   Four runs with glucose, according to the Cori technique, in which 4 cc. of a 60 per cent solution are administered by stomach tube, were made and the

TABLE I

Glycogen Storage with Mannitol and Mannitan in Livers of White Rats

Group No.	No. of rats	Food ingested		Liver glycogen	Group No.	No. of rats	Food ingested		Liver glycogen
		Type	Amount				Type	Amount	
			gm.	per cent				gm.	per cent
1	2	Cacao-butter	20.0	0.06	1	2	Cacao-butter and man- nitan	30.0	
2	2		15.0	0.09	2	2		17.6	
3	2		10 0	0.32	3	2		20.0	
4	2		5.5	0.07	4	2		20 0	
5	3		32.0	0.18	5	2		15.6	
6	4		46.0	0.22	6	1		8.0	0.30
7	4		49.0	0.09	7	1		8.0	
8	4		56.0	0.03	8	1		6.5	
9	3		49.0	0.17	9	1		9.0	0.21
					10	1		7.5	
Average...............				0.14	11	1		8.0	0 23
					12	1		10.0	
1	2	Cacao-butter and man- nitol	20.5	1.80	13	2		11 8	0 09
2	2		23.2	1.00	14	2		13.0	
3	2		23.5	1.60	15	2		16.5	
4	2		19.7	0.62	16	2		13.5	
5	2		22.4	1.60	17	2		13.5	
6	2		14.5	0.69	18	2		21.0	0 09
7	2		17.5	0.74	19	2		15.5	0.07
8	2		16.5	1.22	20*	1		6.5	
9	2		13.4	1.77	21*	1		7 5	
Average...............				1.23					

* Experiments on Groups 20 and 21 were conducted with mannitan prepared by the method of Berthelot (13) which consists essentially of dehydrating mannitol by prolonged boiling under a reflux condenser with several volumes of concentrated hydrochloric acid.

respiratory quotients were 0.888, 0.914, 0.838, and 0.883 with a mean of 0.881.   The technique employed with glucose was used with mannitol.   Owing to the insolubility of this compound, 5 cc. of a 25 per cent solution were administered.   The results of these experiments are shown in Table II.

<div align="center">TABLE II</div>

Respiratory Quotient during Fasting and after Mannitol or after Mannitan

	Weight of rat	O₂	R.Q.
	gm	*gm.*	
Fasting......................	170	1.130	0.716
Mannitol....................	175	0.845	0.727
Fasting......................	160	0.790	0.744
Mannitol...................	165	0 782	0.705
Fasting.............	150	0.875	0.747
Mannitol........... ..	155	0 755	0.755
Fasting......................	205	1.019	0.728
Mannitol................. ..	210	0.823	0.713
Fasting....................	170	0 907	0.735
Mannitol..................	175	0 773	0.731
Mean, fasting........................			0 734
" mannitol			0.726
Fasting..	182	0.834	0.720
Mannitan...	187	0.677	0.770
Fasting..	150	0.750	0.705
Mannitan	155	0 511	0.778
Fasting....	147	0.783	0.720
Mannitan..........	152	0.613	0.740
Fasting.................	175	0 814	0.714
Mannitan........	180	0 577	0.782
Fasting.........	180	0 792	0.743
Mannitan	185	0.564	0 826
Fasting..........	210	1.144	0.728
Mannitan.............	215	0.918	0.747
Fasting......................	123	0.601	0.675
Mannitan..................	128	0.570	0.765
Fasting......	173	0.643	0.713
Mannitan..............	178	0.579	0.758
Fasting......................	185	0 638	0 695
Mannitan........	190	0.739	0.763
Fasting......................	173	0.716	0.737
Mannitan........	178	0.571	0 795
Fasting.................	160	0.675	0.725
Mannitan..................	165	0.683	0.716
Fasting.................. ...	175	0.829	0.735
Mannitan*..................	180	1.266	0.758
Mean, fasting...			0.718
" mannitan...			0.766

* These values represent a 5 hour period after the administration of mannitan.

The results shown in Table II indicate that over a $2\frac{1}{2}$ hour period the ingestion of mannitol does not significantly affect the respiratory quotient of a fasting white rat. The fasting quotients of these rats were somewhat higher than in the control series, indicating the advisability of the procedure herein employed of determining the fasting respiratory quotient on each animal before administering the substance under investigation. The difference between the two means of 0.008 (lower with mannitol) is considered insignificant by the authors and the experiment is interpreted to mean that under these conditions mannitol does not influence the respiratory quotient.

The next step in this investigation concerned the effect of mannitan on the respiratory quotient. The solubility of mannitan in water permitted the use of a 40 to 50 per cent solution. A volume of 4 cc. was administered. The results of these experiments also are shown in Table II.

Following this a series of respiratory quotients was determined on a group of fasting rats after the administration of mannitan as described previously. The fasting values were not determined prior to the administration of mannitan. These respiratory quotients were 0.782, 0.746, 0.750, 0.797, 0.729, and 0.774 with a mean of 0.763. The measurements of the respiratory quotients indicate that although the ingestion of mannitol does not raise the respiratory quotient, mannitan given under the same experimental conditions causes a slight but significant rise. Having observed in feeding experiments the depletion of liver glycogen in the feeding of mannitan, the attention of the authors was centered next on an explanation for the increased respiratory quotient after the ingestion of mannitan. It seemed to resolve itself into two possible explanations; *viz.*, the direct utilization of mannitan or the utilization of glycogen mobilized by its presence in the body.

To determine the answer to this question a series of tissue glycogen determinations was made on rats fasted and fed pure fat as previously described. A second series of experiments was conducted on rats fed fat plus mannitan under the conditions set forth in the liver glycogen feeding experiments. The tissue glycogen was determined by rapidly killing the rats by exsanguination and removing the liver and alimentary tract from the posterior end of the esophagus to the anus. The carcass was rapidly

weighed and cut into ten fragments by means of a cleaver.   Each portion was transferred immediately to 100 cc. of a 60 per cent potassium hydroxide solution heated on a boiling water bath. The entire operation consumed not longer than 3 minutes.   Hence, freezing of the tissues by means of solid carbon dioxide as suggested by Cori and Cori (15) seems to be unnecessary.   After a period of 3 hours, the bones were removed by straining and the solution was made up to 250 cc.   The glycogen was determined on a 50 cc. aliquot by the methods previously described.

The results with the control rats of this experiment show that the percentage of tissue glycogen was 0.109, 0.101, 0.133, 0.165, 0.131, 0.130, 0.172, and 0.212 with a mean of 0.144.   With mannitan-fed rats, the following percentages of tissue glycogen were obtained: 0.148, 0.152, 0.133, 0.191, 0.138, 0.180, 0.133, 0.180, 0.194, and 0.205 with a mean of 0.164.   These results indicate that when mannitan is fed according to the foregoing experimental procedure, the tissue glycogen of the rats shows no significant difference from the tissue glycogen content of rats fed cacao-butter alone.

During the respiratory quotient experiments, the injection by stomach tube of 2.5 gm. of mannitan seemed to exert a narcotic effect on certain of the animals, particularly those of relatively small body weight.   This narcotic effect was observed on white rats injected intraperitoneally with mannitol solutions by Macht and Ting (16).   Narcotic action was not observed during the course of the feeding experiments, although mannitol and mannitan each produced marked diarrhea.

In the belief that perhaps the mobilization of the tissue and liver glycogen was associated with the narcotic action of mannitan as manifested under the conditions of the respiratory experiments, the following procedure was employed.

Male rats of reasonably uniform weight were fasted for 48 hours. They were then given 2.5 gm. of mannitan by stomach tube by the technique previously described.   The tissue glycogen was determined as set forth in the foregoing descriptions.   The results of this experiment are shown in Table III.

These results present an entirely different picture from that obtained when mannitan is fed.   When injected by stomach tube, there appears to be a diminution of tissue glycogen to approxi-

mately one-third the normal fasting value. The mobilization of the tissue glycogen by mannitan when given by stomach tube presents a possible explanation of its capacity to raise the respiratory quotient.

Acute Toxicity—The narcotic effect of mannitol and mannitan observed in these experiments prompted the determination of the acute toxicity of the two compounds. It seemed of special interest to observe, since the production of the anhydride grouping so radically changed the metabolism of the compound, whether or not a change in toxicity was concomitant with the change in its metabolism. The mannitol and mannitan were

TABLE III

Tissue and Liver Glycogen (Mannitan by Stomach Tube)

Weight of rat	Weight of tissue	Glycogen in tissues	
gm.	*gm.*	*gm.*	*per cent*
160	125	0.068	0.054
200	165	0 183	0.111
155	125	0 023	0.018
140	110	0.031	0.028
185	150	0 070	0 046
150	115	0 029	0 025
165	130	0.092	0.071
150	115	0.067	0.058
150	118	0 044	0.037
145	115	0.040	0.035
Mean....................			0 048

administered by stomach tube as previously described. The M.L.D. of mannitol by stomach tube for white rats (fifteen experiments) is approximately 1.3 gm. per 100 gm. of body weight. The M.L.D. of mannitan by stomach tube for white rats (nine experiments) is approximately the same as that for mannitol. The animals showed no evidence of convulsions prior to death. Death is apparently due to respiratory failure, as heart action continued after the cessation of respiration.

Fate of Mannitan in Body—To determine the presence or absence of mannitan in the urine of a group of rats fed mannitan and cacao-butter, the combined urine from eight rats (about 75 cc.)

was evaporated to dryness on a water bath, at a temperature not exceeding 75°. The residue, which was syrupy, was dried to a constant weight at 110° and mixed with washed sea sand. This mixture was extracted with absolute alcohol in a Soxhlet extractor. The alcohol was evaporated and the residue tested for mannitan. About 12 gm. of material were obtained, which coincided in physical properties and responded to the qualitative tests for mannitan. The residue gave a characteristic blue color when gently warmed with Froehde's reagent. It decolorized diluted acidified solutions of potassium permanganate in the cold. Neither glucose nor mannitol responds to this test. This experiment indicates that much of the mannitan ingested by the white rat appears unchanged in the urine.

Mannitan and Insulin Shock—The combatting of insulin shock with various carbohydrates and related compounds has been investigated by various workers. Noble and Macleod (17) found that levulose, galactose, and maltose provided temporary relief in insulin shock in rabbits and that glucose and mannose provided permanent relief of symptoms. Voegtlin *et al.* (6), as was stated previously, observed the failure of mannitol to relieve hypoglycemia. Accordingly the authors studied the influence of mannitan on insulin hypoglycemia. 5 units of insulin per 100 gm. of mouse were injected and at the beginning of the convulsive period 1.4 cc. of a 15 per cent mannitan solution per 100 gm. of mouse were injected intraperitoneally. When glucose was employed the same procedure was followed.

In thirteen experiments with mice, mannitan failed to relieve permanently hypoglycemia produced by insulin when administered in doses found to be effective when glucose was used.

Mannitol, Mannitan, and Blood Sugar Level—The influence of the ingestion of mannitol on the blood sugar level of humans was studied in a preliminary manner by Field (5). In the light of the foregoing findings in this study, it was thought to be of sufficient interest to study the influence of mannitol and its anhydride mannitan on the blood sugar level of rabbits. Fourteen rabbits weighing about 2 kilos were fasted for 48 hours. Their fasting blood sugar levels were determined by the Shaffer-Hartmann (12) method. 4 gm. per kilo respectively of glucose, mannitol, and mannitan were administered by stomach tube and at $\frac{1}{2}$ hour

intervals subsequent determinations were made. A summary
of the results obtained is shown in Table IV.

TABLE IV

Summary of Results.   Glucose, Mannitol, and Mannitan

| | Mg per 100 cc. blood | | | | | |
	Fasting	½ hr.	1 hr.	1¼ hrs	2 hrs.	3 hrs.
Glucose	97	179	255	246	243	197
Mannitol	105	159	155	154	119	143
Mannitan..	101	111	103	95	106	111

DISCUSSION

In this investigation a series of twenty-six fasting respiratory
quotients for white rats of various body weights has been reported.
The mean of this series is 0.717. The probable error of a single
determination of this series calculated by the formula, probable
error $= \pm 0.6745 \sqrt{\Sigma(v^2)/(n-1)}$ is ± 0.012. If one considers a
significant difference to be of the order of magnitude of 3 probable
error or ± 0.036, it becomes evident that the mean 0.766 re-
spiratory quotient obtained after mannitan ingestion represents a
significant rise in the respiratory quotient.

It is therefore concluded from the gaseous exchange experiments
that mannitan produces a slight but definite increase in the respir-
atory quotient. The question as to whether this is due to the
catabolism of mannitan or the mobilization of tissue glycogen and
its catabolism may be considered in the light of the following facts.
The data assembled in Table III show that when mannitan is
administered by stomach tube under the conditions of the experi-
ment there was a marked diminution of tissue glycogen. The
mean difference in tissue glycogen observed is 0.12 per cent.
Setting the average weight of the rat tissue at 125 gm., 0.15 gm.
of glucose becomes available for catabolism after the administra-
tion of mannitan. Theoretically the oxidation of mannitan alone
would raise the respiratory quotient to 0.923. It would require
the oxidation of relatively larger amounts of mannitan than
glucose to raise the respiratory quotient to 0.766. In view of the
facts that the injection of mannitan makes available the glucose
for oxidation and that much of the mannitan can be recovered

unchanged in the urine, the authors are inclined to believe that the increase in repiratory quotient is due to the catabolism of glucose and not of mannitan.

An examination of the oxygen consumption of the fasting rats whose respiratory quotient was determined reveals that in twenty-six determinations an average of 0.174 gm. of oxygen was consumed per 100 gm. of rat each hour. An inspection of the oxygen consumption of rats after the injection of mannitan shows (average of nineteen) 0.139 gm. of oxygen consumed per 100 gm. of rat each hour or a diminution of 20 per cent. As the compound produces death by respiratory failure, the diminished oxygen consumption of the animals is due likely to depression of the respiratory center. An examination of the data in Table II shows that the injection of mannitol likewise produces a diminution in the oxygen consumption.

SUMMARY

1. The removal of 1 molecule of water from mannitol with the formation of its anhydride mannitan destroys the capacity of the former compound to be stored as glycogen in the liver of white rats.

2. Mannitol does not significantly affect the respiratory quotient of white rats. Mannitan produces a slight but definite rise in the respiratory quotient.

3. There is no significant difference in the toxicities of the compounds when injected by means of a stomach tube.

4. Mannitan is ineffective in relieving insulin shock.

5. Mannitan does not affect the fasting blood sugar level of rabbits when administered orally. Mannitol produces a slight but significant rise.

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BUFFER INTENSITIES OF MILK AND MILK CONSTITUENTS

II. BUFFER ACTION OF CALCIUM PHOSPHATE

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The extent and manner in which casein participates in the buffer action of milk have been discussed in Paper I of this series (7). In that paper it was also shown that the buffer intensities of hydrochloric acid whey vary considerably with variations in hydrogen ion concentration and are of sufficient magnitude to be considered of importance equal to that of the buffer intensities of casein. The phosphates and citrates of milk, since their concentrations are comparatively large, should be the salt systems of major importance in this buffer action.

Evaluation of the part played by these constituents is complicated by the facts that these salts are only partially in crystalloidal solution, that the ratio between colloidal and crystalloidal forms is apparently variable, and that calcium ions are involved in the buffer action. This paper deals with the calcium phosphate system only.

The calcium phosphate equilibria in their component relationship in the ionic equilibria of blood, and particularly in respect to their influence on calcium concentration, have been considered by Kugelmass and his coworkers (3, 4). Rüdiger and Wurster (5) have investigated the effects of addition of calcium salts on the hydrogen ion concentration and rennet coagulation of milk. Buffering in homogeneous and heterogeneous systems has been covered in a general theoretical way by Täufel and Wagner (6). Klinke (2) has outlined in mathematical detail the behavior of the simpler, typical buffer systems, stopping just short of the type exemplified by calcium phosphate solutions. A more extensive

list of references may be obtained by consulting the five articles specifically mentioned.

In this paper, Klinke's method of formulation is applied to the system of calcium phosphate solution in equilibrium with solid dicalcium phosphate and to the system of calcium phosphate solution in equilibrium with solid tricalcium phosphate. Results obtained by substitution in these formulas of values equivalent to the concentrations of calcium and phosphate in acid whey have been compared with results from values obtained by titration of solutions of the same concentration. In the evaluation of the equations, the values for dissociation and solubility product constants have been taken from work of Holt, La Mer, and Chown (1). These seemed to be the most highly accurate values in 1928, when most of the work of this paper was done. Substitution of more recently determined values would not appreciably affect the mathematical results of this paper.

Derivation of Equations for Hydrogen Ion Buffer Intensity of Calcium Phosphate Solutions in Equilibrium with Solid Dicalcium Phosphate—For the purposes of convenience and of economy of space

P_3 represents concentration of $PO_4^{\equiv}$ (in mols per liter)

P_2 " " " $HPO_4^{=}$

P_1 " " " $H_2PO_4^{-}$

P " " " H_3PO_4

S " the sum of the concentrations of all forms of phosphoric acid in solution

H represents concentration of hydrogen ions

pH " log $1/H$

OH " concentration of hydroxyl ions

Ca " " " calcium "

B represents the number of mols of completely dissociated monobasic acid or mono-acid base added per liter of total volume

k_1, k_2, k_3, represent, respectively, the first, second, and third dissociation constants of phosphoric acid

K_D represents the solubility product of dicalcium phosphate

w represents the function, $k_1k_2k_3 + k_1k_2H + k_1H^2 + H^3$

In the subsequent calculations, $k_1 = 1 \times 10^{-2}$, $k_2 = 1 \times 10^{-7}$, $k_3 = 4 \times 10^{-13}$, $K_D = 1 \times 10^{-6}$, and K_T, solubility product of tricalcium phosphate, $= 3.61 \times 10^{-33}$ (Holt *et al.* (1)).

In the derivation of an equation for dB/dpH, the buffer index, the term $d\mathrm{P}_2/d\mathrm{H}$, expressing the rate of change of concentration of secondary phosphate ion with change of concentration of hydrogen ion, appeared. In the following development, a rational expression for the derivative of the concentration of secondary phosphate ion is first obtained, and subsequently the buffer index is derived, since this seemed to be the most logical order from the standpoint of smoothness and increasing complexity.

Since, by hypothesis, the secondary phosphate ion and calcium ion may disappear from solution by precipitation as dicalcium phosphate

$$\text{(1)} \qquad \mathrm{P}_2 = f(\mathrm{Ca})$$

This function is expressed by the solubility product

$$\text{(2)} \qquad \mathrm{Ca} \cdot \mathrm{P}_2 = K_\mathrm{D}$$

The concentration of the secondary phosphate ion is also a function of the hydrogen ion concentration and of the whole concentration of phosphate in solution.

$$\text{(3)} \qquad \mathrm{P}_2 = f\,(\mathrm{H},\,S)$$

This function may be derived from the expressions for the successive dissociations of phosphoric acid, as follows:

(4) $\mathrm{HP}_1/\mathrm{P} = k_1$ \qquad (4,a) $\mathrm{P} = \mathrm{HP}_1/k_1$

(5) $\mathrm{HP}_2/\mathrm{P}_1 = k_2$ \qquad (5,a) $\mathrm{P}_1 = \mathrm{HP}_2/k_2$

(6) $\mathrm{HP}_3/\mathrm{P}_2 = k_3$ \qquad (6,a) $\mathrm{P}_3 = k_3\mathrm{P}_2/\mathrm{H}$

Substitution of Equation 5,a in Equation 4,a gives

$$\text{(4,b)} \qquad \mathrm{P} = \mathrm{H}^2\mathrm{P}_2/k_1k_2$$

By convention

$$\text{(7)} \qquad S = \mathrm{P} + \mathrm{P}_1 + \mathrm{P}_2 + \mathrm{P}_3$$

By substitution in Equation 7

$$\text{(8)} \qquad S = (\mathrm{H}^2\mathrm{P}_2/k_1k_2) + (\mathrm{HP}_2/k_2) + \mathrm{P}_2 + (k_3\mathrm{P}_2/\mathrm{H})$$

By solving for P_2

(9)
$$P_2 = \frac{k_1k_2HS}{k_1k_2k_3 + k_1k_2H + k_1H^2 + H^3} = \frac{k_1k_2HS}{w}$$

By comparison with Equation 3

(10)
$$f(H) = k_1k_2H/w \quad \text{and} \quad f(S) = S$$

Differentiation of Equation 3 with respect to H gives

(11)
$$\frac{dP_2}{dH} = \frac{\partial f(H)}{\partial H} \cdot f(S) \cdot \frac{dH}{dH} + \frac{\partial f(S)}{\partial S} \cdot f(H) \cdot \frac{dS}{dH}$$

Since S can vary only by precipitation or solution of $CaHPO_4$,

(12)
$$dS = dCa$$

By differentiation of Equation 2

(13)
$$dCa = - (K_D/P_2^2) \cdot dP_2$$

By substitution of Equation 9 in Equation 13

(14)
$$dCa = - (K_D w^2/k_1^2 k_2^2 H^2 S^2) \cdot dP_2$$

By substitution of this value of dCa for dS, S for $f(S)$, and 1 for dH/dH and $\partial f(S)/\partial S$, Equation 11 becomes

(15)
$$\frac{dP_2}{dH} = \frac{\partial f(H)}{\partial H} \cdot S - \frac{K_D w^2 \cdot f(H)}{k_1^2 k_2^2 H^2 S^2} \cdot \frac{dP_2}{dH}$$

By solving for dP_2/dH, Equation 15 becomes

(16)
$$\frac{dP_2}{dH} = \frac{\partial f(H)}{\partial H} \cdot \frac{k_1^2 k_2^2 H^2 S^3}{k_1^2 k_2^2 H^2 S^2 + K_D w^2 \cdot f(H)}$$

By differentiation of Equation 10,

(17)
$$\partial f(H)/\partial H = (k_1k_2(k_1k_2k_3 - k_1H^2 - 2H^3))/w^2$$

By substitution of values of Equations 10 and 17 in Equation 16

(18)
$$\frac{dP_2}{dH} = \frac{k_1^2 k_2^2 HS^2(k_1k_2k_3 - k_1H^2 - 2H^3)}{w^2(k_1k_2HS^2 + K_D w)}$$

In any solution containing the ions derived only from calcium phosphate and water, the ionic equivalence may be expressed in terms of molal concentrations of the participating ions, as follows:

(19) $$H + 2Ca = OH + P_1 + 2P_2 + 3P_3$$

If then the molal concentration of acid is increased by B mols of completely dissociated acid, each of the concentrations of ions is changed and the ionic equivalence may be represented by a new equation

(20) $$H + 2Ca = OH + P_1 + 2P_2 + 3P_3 + B$$

By rearrangement and substitution of values from Equations 2, 5,a, and 6,a

(21) $$B = H - OH + (2K_D/P_2) - 2P_2 - (HP_2/k_3) - (3k_3P_2/H)$$

By differentiation with respect to H

(22) $$\frac{dB}{dH} = \frac{dH}{dH} - \frac{dOH}{dH} - \frac{dP_2}{dH}\left(\frac{2K_D}{P_2{}^2} + 2 + \frac{H}{k_3} + \frac{3k_3}{H}\right) - \frac{P_2}{k_3} + \frac{3k_3P_2}{H^2}$$

$(dH/dH) - (dOH/dH)$ is comparatively so small between pH 3 and 9 that it may be omitted. By substitution of the values of Equations 9 and 18

(23) $$\frac{dB}{dH} = \frac{3k_1k_2k_3S - k_1H^2S}{wH} - \frac{S(k_1k_2k_3 - k_1H^2 - 2H^3)}{w^2H(k_1k_2HS^2 + K_Dw)} \times$$

$$(2K_Dw^2 + 2k_1{}^2k_2{}^2H^2S^2 + k_1{}^2k_2H^3S^2 + 3k_1{}^2k_2{}^2k_3HS^2)$$

By definition

(24) $$dH/dpH = -2.303H$$

Multiplication of Equation 23 by Equation 24 gives

(25) $$dB/dpH = \frac{2.303S}{w} \times$$

$$\frac{(k_1k_2k_3 - k_1H^2 - 2H^3)\ (2K_Dw^2 + 2k_1{}^2k_2{}^2H^2S^2 + k_1{}^2k_2H^3S^2 + 3k_1{}^2k_2{}^2k_3HS^2)}{w(k_1k_2HS^2 + K_Dw)} - 3k_1k_2k_3 + k_1$$

Thus in Equations 23 and 25 the effect on the hydrogen ion concentration of additions of acid or base to calcium phosphate solutions in equilibrium with solid dicalcium phosphate is expressed in terms of two directly determinable variables—hydrogen ion concentration and total soluble phosphate concentration.   If the solubility of dicalcium phosphate were of the order of that of sodium phosphate, the buffering would be purely homogeneous, the first term in the parentheses of Equation 22 would become 0 and S would become a constant.   Equation 25 would then become

$$(26) \quad \frac{dB}{d\mathrm{pH}} = - \frac{2.303k_1\mathrm{H}S}{w^2} \, (k_1k_2{}^2k_3 + 4k_1k_2k_3\mathrm{H} + k_1k_2\mathrm{H}^2 + 9k_2k_3\mathrm{H}^2 + 4k_2\mathrm{H}^3 + \mathrm{H}^4)$$

By methods similar to those used in deriving Equation 18, the hydrogen ion buffering effects of additions of calcium salts or of phosphates may be formulated

$$(27) \quad \frac{d\mathrm{Ca}}{d\mathrm{H}} = \frac{dS}{d\mathrm{H}} = - \frac{K_\mathrm{D}S(k_1k_2k_3 - k_1\mathrm{H}^2 - 2\mathrm{H}^3)}{\mathrm{H}(K_\mathrm{D}w + k_1k_2\mathrm{H}S^2)}$$

$$(28) \quad \frac{d\mathrm{Ca}}{d\mathrm{pH}} = \frac{dS}{d\mathrm{pH}} = \frac{2.303K_\mathrm{D}S(k_1k_2k_3 - k_1\mathrm{H}^2 - 2\mathrm{H}^3)}{K_\mathrm{D}w + k_1k_2\mathrm{H}S^2}$$

Values of S to be substituted in Equations 18, 23, 25, 27, and 28 may be calculated by assuming all calcium in solution to be ionized and by postulating a definite relationship between soluble calcium and phosphate.   The concentration of phosphate in acid whey is approximately 0.0211 M; that of calcium, 0.03165 M.

$$(29) \qquad\qquad \mathrm{Ca} = S + 0.01055$$

By elimination of Ca and P_2 among Equations 2, 9, and 29

$$(30) \qquad S = \sqrt{(K_\mathrm{D}w/k_1k_2\mathrm{H}) + 0.00002783} - 0.005275$$

Calculation of Equations for Hydrogen Ion Buffer Intensity of Calcium Phosphate Solutions in Equilibrium with Solid Tricalcium Phosphate—The notation is the same as in the previous formulation, with the addition that K_T represents the solubility product of $Ca_3(PO_4)_2$.

$$(31) \qquad\qquad P_3 = f(\mathrm{Ca})$$

This function is expressed by the solubility product

(32) $\quad Ca^3 \cdot P_3{}^2 = K_T$ (33) $\quad P_3 = f(H,S)$

From Equations 4, 5, and 6

(34) $\quad P_2 = HP_3/k_3$ (35) $\quad P_1 = H^2P_3/k_2k_3$ (36) $\quad P = H^3P_3/k_1k_2k_3$

By substitution of these values in Equation 7

(37) $\qquad P_3 = \dfrac{k_1k_2k_3S}{k_1k_2k_3 + k_1k_2H + k_1H^2 + H^3} = \dfrac{k_1k_2k_3S}{w}$

By comparison with Equation 33

(38) $\qquad f(H) = k_1k_2k_3/w \quad \text{and} \quad f(S) = S$

By differentiation of Equation 33

(39) $\qquad \dfrac{dP_3}{dH} = \dfrac{\partial f(H)}{\partial H} \cdot f(S) \cdot \dfrac{dH}{dH} + \dfrac{\partial f(S)}{\partial S} \cdot f(H) \cdot \dfrac{dS}{dH}$

Since S can vary only by precipitation or solution of $Ca_3(PO_4)_2$,

(40) $\qquad\qquad dS = 2/3 \cdot dCa$

By differentiation of Equation 32 and substitution of Equation 37,

(41) $\qquad dCa = -\ 2/3 \cdot w/k_1k_2k_3S \sqrt[3]{K_Tw^2/k_1{}^2k_2{}^2k_3{}^2S^2} \cdot dP_3$

By substitution in Equation 39,

(42) $\qquad \dfrac{dP_3}{dH} = \dfrac{\partial f(H)}{\partial H} \cdot S - \dfrac{4}{9} \cdot \dfrac{1}{S} \sqrt[3]{\dfrac{K_Tw^2}{k_1{}^2k_2{}^2k_3{}^2S^2}} \cdot \dfrac{dP_3}{dH}$

By solving for dP_3/dH, Equation 42 becomes

(43) $\qquad \dfrac{dP_3}{dH} = \dfrac{\partial f(H)}{\partial H} \cdot \dfrac{9S^2 \sqrt[3]{k_1{}^2k_2{}^2k_3{}^2S^2}}{9S \sqrt[3]{k_1{}^2k_2{}^2k_3{}^2S^2} + 4 \sqrt[3]{K_Tw^2}}$

Differentiation of Equation 38 gives

(44) $\qquad \partial f(H)/\partial H = -\ k_1k_2k_3(k_1k_2 + 2k_1H + 3H^2)/w^2$

By substitution in Equation 43

(45) $\qquad \dfrac{dP_3}{dH} = -\ \dfrac{9k_1k_2k_3S^2 \sqrt[3]{k_1{}^2k_2{}^2k_3{}^2S^2(k_1k_2 - 2k_1H - 3H^2)}}{w^2 \left(9S \sqrt[3]{k_1{}^2k_2{}^2k_3{}^2S^2} - 4 \sqrt[3]{K_Tw^2}\right)}$

By substitution in the ionic equivalence Equation 20 of values of Equations 32, 35, and 34

$$(46) \qquad B = H - OH + \frac{2\sqrt[3]{K_T}}{\sqrt[3]{P_3{}^2}} - \frac{H^2 P_3}{k_2 k_3} - \frac{2HP_3}{k_3} - 3P_3$$

By differentiation with respect to H, $(dH/dH) - (dOH/dH)$ being omitted,

$$(47) \qquad \frac{dB}{dH} = -\frac{dP_3}{dH}\left(\frac{4\sqrt[3]{K_T}}{3\sqrt[3]{P_3{}^5}} + \frac{H^2}{k_2 k_3} + \frac{2H}{k_3} + 3\right) - \frac{2HP_3}{k_2 k_3} - \frac{2P_3}{k_3}$$

By substitution of values from Equations 37 and 45

$$(48) \qquad \frac{dB}{dH} = \frac{S}{w}\left[\frac{3(k_1 k_2 + 2k_1 H + 3H^2)}{w(9S\sqrt[3]{k_1{}^2 k_2{}^2 k_3{}^2 S^2} + 4\sqrt[3]{K_T w^2})} \times\right.$$

$$(4w\sqrt[3]{K_T w^2} + 3k_1 H^2 S\sqrt[3]{k_1{}^2 k_2{}^2 k_3{}^2 S^2} + 6k_1 k_2 H S \times$$

$$\left.\sqrt[3]{k_1{}^2 k_2{}^2 k_3{}^2 S^2} + 9k_1 k_2 k_3 S\sqrt[3]{k_1{}^2 k_2{}^2 k_3{}^2 S^2}) - 2k_1 H - 2k_1 k_2\right]$$

Multiplication of Equation 48 by Equation 24 gives

$$(49) \qquad \frac{dB}{dpH} = -\frac{2.303 HS}{w}\left[\frac{3(k_1 k_2 + 2k_1 H + 3H^2)}{w(9S\sqrt[3]{k_1{}^2 k_2{}^2 k_3{}^2 S^2} + 4\sqrt[3]{K_T w^2})} \times\right.$$

$$(4w\sqrt[3]{K_T w^2} + 3k_1 H^2 S\sqrt[3]{k_1{}^2 k_2{}^2 k_3{}^2 S^2} + 6k_1 k_2 H S\sqrt[3]{k_1{}^2 k_2{}^2 k_3{}^2 S_2} +$$

$$\left.9k_1 k_2 k_3 S\sqrt[3]{k_1{}^2 k_2{}^2 k_3{}^2 S^2}) - 2k_1 H - 2k_1 k_2\right]$$

If, in this equation, the terms representing the heterogeneous buffering be made equal to 0, this becomes identical with Equation 26.

The following values may be derived by a process analogous to that used in deriving Equation 45

$$(50) \qquad \frac{dS}{dH} = \frac{4S\sqrt[3]{K_T}(k_1 k_2 + 2k_1 H + 3H^2)}{\sqrt[3]{w}\,(4\sqrt[3]{K_T w^2} + 9S\sqrt[3]{k_1{}^2 k_2{}^2 k_3{}^2 S^2})}$$

$$(51) \qquad \frac{dS}{dpH} = -\frac{9.212 HS\sqrt[3]{K_T}(k_1 k_2 + 2k_1 H + 3H^2)}{\sqrt[3]{w}\,(4\sqrt[3]{K_T w^2} + 9S\sqrt[3]{k_1{}^2 k_2{}^2 k_3{}^2 S^2})}$$

The corresponding values involving dCa may be obtained from the above by multiplying by 3/2.

Values of S to be substituted in the above equations may be calculated on the same assumptions as in the previous case, except that

(52) $$Ca = 3/2S$$

From Equations 32, 37, and 52

(53) $$S = \sqrt[3]{8K_T w^2/27 k_1^2 k_2^2 k_3^2}$$

These equations are directly applicable only in so far as the constants involved are definitely established for the conditions being studied. The most serious difficulty is in the variation of solubility products with values of ionic strength. Holt, La Mer, and Chown (1) have considered these variations and have plotted the values of solubility products of dicalcium phosphate and tricalcium phosphate against ionic strength. They find that the effect is greater on the product for tricalcium phosphate than on that for dicalcium phosphate, a fact of considerable significance in deciding which should be the stable solid phase under any given set of conditions. Since the calculation of values of ionic strength in biological fluids is highly uncertain on the basis of available data, and since true equilibria in most of such fluids are attained only after an exceedingly long time, the equations of this paper have been applied to calculations of equilibrium values on the hypothetical basis of no ionic strength or other complicating influences. The disturbing factors are then discussed in so far as they would affect the results in actual application.

Since the author is chiefly interested in applications to milk and to fluids derived from milk, calculations were made on a solution containing initially 0.0211 mol of total phosphate and 0.03165 mol of calcium per liter. Concentrations and buffer indices calculated for pH values at which these amounts would not saturate the solution are, however, saturation values and consequently are for concentrations greater than these amounts would furnish. In Table I are given values selected for their special interest from a large number calculated. The equations employed in the calculation of these values are Nos. 30, 9, 4,*b*, 5,*a*, 6,*a*, 2, 26; and 28, 52 37, 36, 35, 34, 32, 49, and 51.

TABLE I

Effect of Changing Hydrogen Ion Concentration on Concentrations of Calcium Ions and Various Forms of Soluble Phosphate

Concentrations are expressed in mols per liter.

pH	S	P	P_1	P_2	P_3	Ca	dB/dCa
		Equilibrium with solid dicalcium phosphate					
3.0	9.96×10^{-2}	9.08×10^{-2}	9.08×10^{-2}	9.08×10^{-6}	3.63×10^{-18}	1.10×10^{-1}	1.18
4.0	2.69×10^{-2}	2.66×10^{-2}	2.66×10^{-2}	2.66×10^{-5}	1.06×10^{-13}	3.75×10^{-2}	1.03
5.0	6.09×10^{-3}	6.02×10^{-3}	6.02×10^{-3}	6.02×10^{-5}	2.41×10^{-12}	1.66×10^{-2}	1.11
6.0	9.50×10^{-4}	8.64×10^{-4}	8.64×10^{-4}	8.64×10^{-5}	3.45×10^{-11}	1.15×10^{-2}	1.17
7.0	1.90×10^{-4}	9.50×10^{-5}	9.50×10^{-5}	9.50×10^{-5}	3.80×10^{-10}	1.07×10^{-2}	1.05
8.0	1.02×10^{-4}	9.50×10^{-6}	9.50×10^{-6}	9.50×10^{-5}	3.71×10^{-9}	1.07×10^{-2}	1.23
9.0	9.50×10^{-5}	9.50×10^{-7}	9.50×10^{-7}	9.50×10^{-5}	3.43×10^{-8}	1.07×10^{-2}	1.08
		Equilibrium with solid tricalcium phosphate					
3.0	5.89×10^{-2}	5.35×10^{-2}	5.35×10^{-2}	5.35×10^{-6}	2.14×10^{-15}	8.84×10^{-2}	5.47
4.0	9.02×10^{-3}	8.93×10^{-5}	8.93×10^{-3}	8.93×10^{-6}	3.57×10^{-14}	1.35×10^{-2}	5.35
5.0	1.43×10^{-3}	1.42×10^{-6}	1.42×10^{-3}	1.42×10^{-5}	5.66×10^{-12}	2.15×10^{-3}	5.33
6.0	2.34×10^{-4}	2.13×10^{-8}	2.13×10^{-4}	2.13×10^{-5}	8.53×10^{-12}	3.51×10^{-4}	5.33
7.0	4.72×10^{-5}	2.36×10^{-10}	2.36×10^{-5}	2.36×10^{-5}	9.44×10^{-11}	7.08×10^{-5}	5.28
8.0	1.48×10^{-5}	1.35×10^{-12}	1.35×10^{-6}	1.35×10^{-5}	5.38×10^{-10}	2.22×10^{-5}	4.86
9.0	5.69×10^{-6}	5.63×10^{-15}	5.63×10^{-8}	5.63×10^{-6}	2.29×10^{-9}	8.54×10^{-6}	4.69

The changes in concentrations of total soluble phosphate, of each of the forms of soluble phosphate, and of calcium ions, are seen to be in general logarithmic in nature in relation to pH changes. The most striking aberrations are at pH values greater
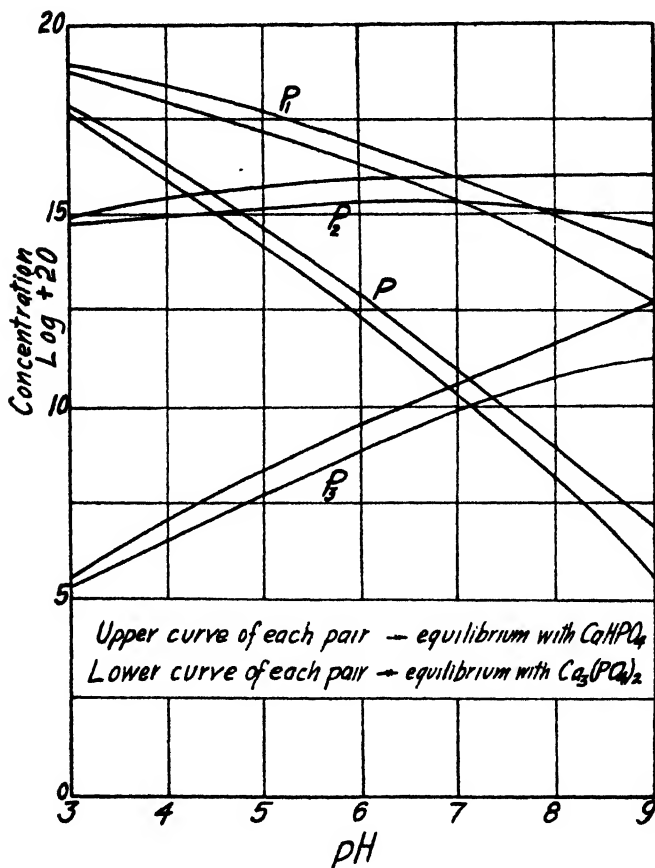
Fig. 1. Effect of changes in hydrogen ion concentration on concentrations of the various forms of soluble phosphate.

than 7.0, where the values for the same item in the two types of equilibria become more widely different. These concentrations vary arithmetically with concentration of hydrogen ions. In Figs. 1 and 2, values are plotted on a logarithmic basis to bring out the relative magnitudes and the relative directions of the changes in

concentrations of each of the forms of soluble phosphate and of calcium ions.

In the uncomplicated case here presented, it is evident that at any pH value saturation with respect to tricalcium phosphate is attained at a lower gross concentration than is saturation with respect to dicalcium phosphate. However, this does not require that tricalcium phosphate shall be the first or only solid to precipi-
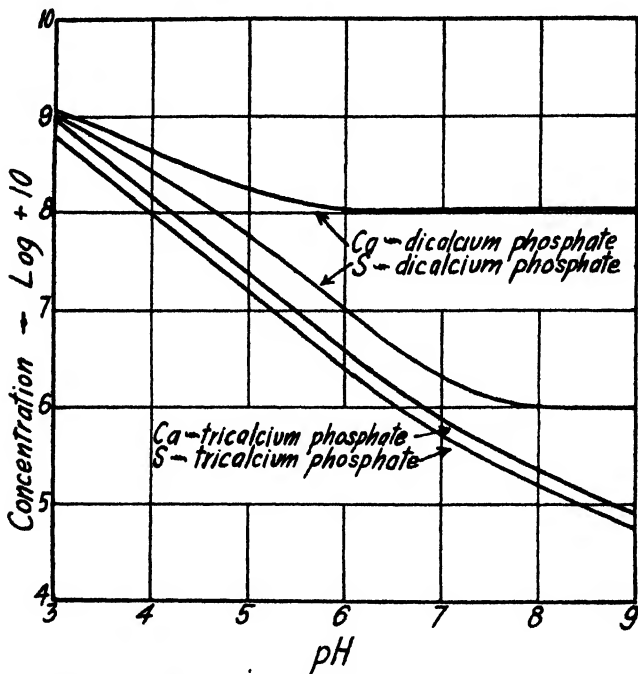
FIG. 2. Effect of changes in hydrogen ion concentration on concentrations of total soluble phosphate and of calcium ions.

tate, because, as is well known, solution equilibrium with respect to tricalcium phosphate is much more slowly attained than it is with respect to dicalcium phosphate. Furthermore, since the equilibrium concentrations are so close to the same values, it would require only a comparatively small alteration in solubility product relations to reverse the order of saturation at pH values less than 7.0. In other words, it is practically possible for alteration of solubility product values to require that dicalcium phos-

phate be the stable solid phase in acid solution, but rather unlikely when dealing with alkaline solutions. Holt, La Mer, and Chown (1) were able to prove by analysis that $CaHPO_4$ is the stable solid phase under certain restricted conditions.
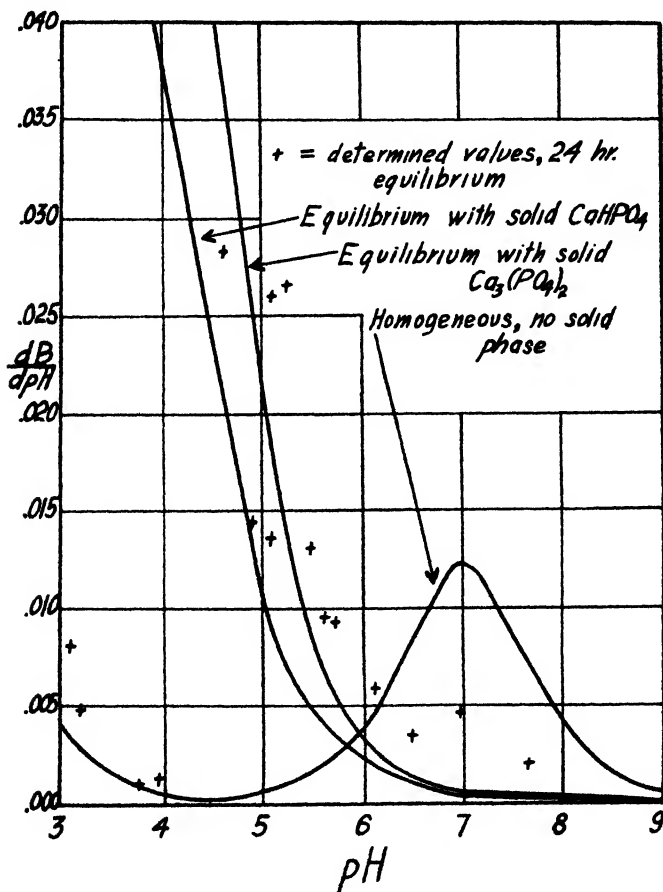
Fig. 3. Effect of changes in hydrogen ion concentration on buffer intensities in calcium phosphate solution.

More information on the calcium phosphate equilibria can be obtained from Fig. 3, in which are plotted hydrogen ion buffer curves for heterogeneous equilibria with di- and with tricalcium phosphates, the corresponding curve for homogeneous equilibria,

and points obtained by actual titration.   Calculations of ion product constants show that, with the concentrations of calcium and phosphate considered, precipitation of tricalcium phosphate should begin at pH 3.8, and precipitation of dicalcium phosphate at pH 4.2.   The buffer indices calculated from the titration follow the course of homogeneous buffering till the pH value is approximately 4.0, but are definitely in the region of heterogeneous buffering at pH 4.6.

It is convenient for purposes of discussion, although fortuitous, that the ionic strength of the solution here considered—approximately 0.085—is practically the same as that calculated by the author as a tentative value for milk (8).   Calculations of ion product constants at pH 4.8 give 4.2×10^{-6} for $Ca \cdot P_2$ and 3.53×10^{-28} for $Ca^3 \cdot P_3^2$, values both very close to the corresponding values of solubility products, read from the plots of Holt, La Mer, and Chown (1), for solutions having ionic strength values of approximately 0.085 ($\sqrt{\mu} = 0.29$).   The fact that heterogeneous buffering appears to begin at a slightly lower pH value than would be predicted from this calculation is probably accounted for by inaccuracies in constants involved in the calculations, by inaccuracies in evaluation of ionic strength, and by neglect of other disturbing effects.   However, it should be evident that one effect of increasing ionic strength is to displace unequally the pH values for saturation with respect to di- and to tricalcium phosphates so that they approach each other and may even reverse their order within certain limits of the acid range and within certain ranges of ratio of calcium to phosphate.

The last column of Table I should be of especial interest to those interested in the relationship in milk among acidity, calcium content, and rennet activity.   These values indicating constancy of ratio between acid addition and change in calcium ion concentration for equilibria involving either the solid di- or tricalcium phosphate are a by-product of these calculations and are offered with full realization that they are not directly applicable to milk itself.

SUMMARY

Generally applicable equations have been derived for the changes in ionic equilibria in calcium phosphate solutions, both on the assumption of the presence of $CaHPO_4$ as solid phase and on

the assumption of $Ca_3(PO_4)_2$ as solid phase.   Calculations on the basis of these equations have been made for initial concentrations of calcium and phosphate equivalent to those in acid whey.   The effect of increased ionic strength on these calculated values has been pointed out for its value in explaining some of the peculiarities in the conduct of whey.   The extent to which these equations and calculations may be applied to milk equilibria is left for subsequent discussion.

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STUDIES OF PHOSPHORUS OF BLOOD

I. THE PARTITION OF PHOSPHORUS IN WHOLE BLOOD AND SERUM, THE SERUM CALCIUM AND PLASMA PHOSPHATASE FROM BIRTH TO MATURITY

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It has been known for many years that the serum inorganic phosphorus of infants and children is normally higher than that of adults (1), that seasonal variations are noticeable (2, 3), and that alterations from the normal are observed in certain diseases of bone (1, 4–7). More recently, it has been demonstrated that, in addition to the seasonal changes in inorganic phosphorus of serum of normal children, distinct changes in level occur during growth (8, 9). Similarly, studies of the lipoid phosphorus of serum have shown that the level of this component alters also during growth, especially through the period of infancy (10, 11).

In view of the detailed studies of the changes in these serum phosphorus compounds during growth, the literature concerning the distribution of phosphorus in whole blood during infancy and childhood is surprisingly scanty (7, 12–17). No data were found for phosphorus partitions of either whole blood or serum during the period of late infancy and early childhood, nor for the period of adolescence. In the studies reported, few investigators have attempted a complete partition of phosphorus, and methods of analysis have differed materially, making comparisons difficult. The more recent studies, wherein modern methods of analysis were used, concern only the period of middle childhood; no study includes the whole period of growth. Because much of the organic phosphorus of the blood is carried in the corpuscles, and because the blood cell picture undergoes marked changes during infancy and childhood, a study of the phosphorus partition of blood covering the entire period from birth to maturity seems desirable.

A summary of the literature concerning phosphorus partitions in blood during growth is given in Table I. Values originally reported as P_2O_5 or H_3PO_4 have been recalculated as mg. of phosphorus per 100 cc. It is evident, from a study of Table I, that existing data are insufficient to establish definitely the normal range of values for phosphorus compounds of blood throughout infancy and childhood.

In the present study, whole oxalated blood and serum were analyzed for total, total acid-soluble, inorganic, and lipoid phosphorus. The ester fraction was determined by difference. Further partition of the ester fraction was not attempted. Serum, rather than plasma, was analyzed in order to make the data more directly comparable with older studies of normal children, and particularly of children with diseases affecting bone. To complete the data for the latter purpose, analyses for serum calcium and plasma phosphatase were included whenever sufficient blood was available.

In order to determine the possibility of seasonal variation in components other than the inorganic phosphorus, the determinations on each age group were spread throughout the year. The cord blood analyses form an exception to this rule, for all of these determinations were made during the late summer. The age grouping and classification of the subjects studied are given in Table II. Because the most marked of the alterations in the cellular components and the known changes in the phosphorus of blood occur before the age of 3 years, almost half the total number of analyses was determined upon this youngest age group. The infants and many of the children were in the hospital only for the purposes of study. No children suffering from bone disease, nor from diabetes, nephritis, or syphilis were included. The adult group was physically well. Nearly all of the older infants and children were ingesting a quart of milk daily. All of the infants and a few of the older children were given cod liver oil. The diets of the adults were not supervised in any way.

All blood samples were taken before breakfast, or, from the infants, just before a feeding. The blood was drawn with as little stasis as possible from the arm or jugular vein of the older infants and children, from the longitudinal sinus of the younger infants, and from the cord vein of the new-born. The serum was separated from the cells promptly, and the analyses carried out

TABLE I

Phosphorus Partition in Blood of Normal Infants and Children.   Data from the Literature

The phosphorus values are given in mg. per 100 cc.

References	Age of subject	No. of analyses	Corpuscles per cent	Whole blood					Plasma or serum					Corpuscles				
				Total P	Lipoid P	Total acid-soluble P	Inorganic P	Ester P	Total P	Lipoid P	Total acid-soluble P	Inorganic P	Ester P	Total P	Lipoid P	Total acid-soluble P	Inorganic P	Ester P
Plass and Tompkins, 1923 (12)	New-born	11							10.7	4 0	5.7	4	51.2					
McKellips, De Young, and Bloor, 1921 (13)	2–26 days	21	44.5	(39.2)	(10 5)	(28.2)	(3 7)	(24 5)	9.4	4.7	5.2	3 02		276	317 7	56.8	4.549	3
Iversen and Lenstrup, 1920 (7)	1–6 mos.*	5		38 7	13 2	23.9			11.7	5.1	6.6							
	2–7 " †	5		34.4	13.4	20 4			10 6	6.2	4.4							
Jones and Nye, 1921 (14)	1–7 mos.	4	32.9	35 2	12 5	23 1	3.1	20.0	11.5	8.6	3.0			82.4	21 1	(63 5)	3 160	4
" " (14)	3–13 yrs.	30	39.0	39 1	11 9	22.6	3 2	19 5	12.3	9.6	2.9			80.6	18 9	(61.5)	3.558	0
Sokolovitch, 1931 (15)	4–12 "	12	33.1	40 1	13.7	26 4	4 2	22.4	13.1	9.1	4.3	3.9		495.1	23 3	(71.9)	4.667	3
Kay and Robison, 1924 (16)	6–12 "	3				22 5	4 2	18 3										
Bomskov, 1932 (17)	7–14 "	9		44 0†	(16 4)‡	26 5	4.2	(22 3)										

Values in parentheses are calculated from data given.

* Breast-fed infants.

† Artificially fed infants.

‡ Average of three determinations only.

with as little delay as possible (18, 19). Serum calcium was determined by the method of Kramer and Tisdall (20), plasma phosphatase by the original method of Kay (21). For cell volume, the Van Allen hematocrit was used, and the oxalated blood centrifuged for 10 minutes at 3500 R.P.M. It is realized that the use of oxalated blood for determinations of cell volume introduces a small error, which, however, is constant throughout the series.

As yet, no method for phosphorus partition seems to have become standard, so the methods used are given in some detail.

TABLE II

Classification of Subjects

Classification	No. of subjects	No. of determinations
New born infants, no disease, weight normal..........	18	18
Infants, 2 wks. to 3 yrs., no disease, weight normal....	22	36
" 2 " " " 3 " with congenital deformities, unoperated*..	14	15
Infants, 2 wks. to 3 yrs., miscellaneous group†..........	3	3
Children, 3 to 15 yrs., no disease, weight normal.......	13	20
" 3 " 15 " with congenital deformities, unoperated*..	13	13
Children, 3 to 15 yrs., miscellaneous group†.............	20	21
Adults, 17 " 28 " speech defects....................	14	17
" 18 " 31 " normal..........................	7	7
Total..	124	150

* Clubbed feet, webbed fingers, wry neck, congenital dislocation of hip.
† Patients with speech defects, disturbances of vision; patients not ill, but in hospital for removal of tonsils and adenoids.

The blood samples were measured into heavy walled Pyrex test-tubes (ignition tubes) graduated at 10 and 20 cc. and were wet-ashed with 10 N sulfuric acid and hydrogen peroxide (phosphorus-free, 30 per cent). The Fiske-Subbarow procedure (22) was used for the development of color. The total quantity of blood available for analysis was necessarily small particularly with the infants and small children. In these latter analyses duplicate determinations of inorganic phosphorus were not made, the control tubes for phosphatase serving as sufficient check. Other phosphorus anal-

yses were carried out in duplicate. The quantities of blood and reagents required for each determination are given below.

Total P—0.1 cc. samples of whole blood or 0.4 cc. of serum are measured into the graduated tubes, and 1 cc. of 10 N sulfuric acid added to each.

Lipoid P—To 0.5 cc. portions of whole blood or 0.6 cc. portions of serum in the graduated tube are added about 8 cc. of Bloor's alcohol-ether mixture (23). Adding the solvent rapidly to the blood in the tube results in the formation of a very fine precipitate. The tubes are placed in boiling water, the contents stirred vigorously for 2 to 3 minutes, cooled, and the volume made up to 10 cc. with alcohol-ether. The mixture is then filtered. 5 cc. samples of the filtrate are evaporated to dryness in a similar tube, and 0.5 cc. of 10 N sulfuric acid added.

Total Acid-Soluble and Inorganic P—To 3 cc. of blood or serum are added 12 cc. of 10 per cent trichloroacetic acid (free from phosphorus); the resulting mixture is filtered. For inorganic phosphorus the customary Fiske-Subbarow procedure is followed, 5 cc. portions of filtrate being used. For total acid-soluble phosphorus, 1 cc. of blood filtrate is mixed with 1 cc. of sulfuric acid (10 N), or 3 cc. of serum filtrate and 0.5 cc. of sulfuric acid are taken. The trichloroacetic acid solution should not be kept longer than 2 weeks.

Heating the tubes in a bath containing saturated calcium chloride solution until the water is driven off, insures against spattering. After charring occurs, the tubes are transferred to a wire rack on a hot-plate, and the digestion is continued until the solutions are colorless. The use of peroxide shortens the period of digestion materially. It is important that the heating be continued until all traces of peroxide are driven off. The tubes are then allowed to cool for an hour or more before the color is developed.

In the development of color in the heated bloods, 2.5 per cent ammonium molybdate in water solution (Molybdate III) is used throughout, together with the customary aminonaphtholsulfonic acid reagent. The final dilution for all tubes containing 0.5 cc. of sulfuric acid (lipoid P, serum total acid-soluble P) is 10 cc.; the contents of the tubes containing 1 cc. of sulfuric acid (total P, total acid-soluble P of whole blood) are diluted to 20 cc. The proportions of reagents added are regulated according to the dilution.

The colors are read against standards containing 0.24 and 0.4 mg. of phosphorus respectively.

DISCUSSION

Lack of space prevents the presentation of the detailed data. The individual determinations are shown graphically in Charts 1
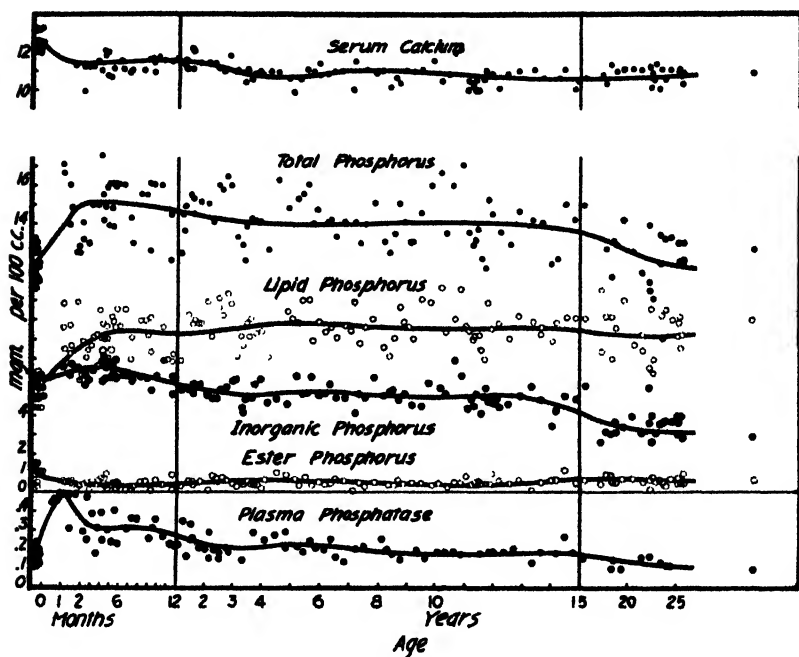
CHART 1. The partition of phosphorus in serum, the serum calcium and plasma phosphatase from birth to maturity.

and 2.   The curves are drawn from the group averages, smoothed. The data for corpuscle phosphorus (Chart 4) were calculated from the group averages for whole blood and serum.

Serum

Inorganic P—Comparatively low values (3 to 5 mg. per 100 cc.) for inorganic phosphorus of serum of new born infants have been reported by several investigators (12, 13, 24–27).   The values

reported here (Chart 1) averaged 5.6 mg. per 100 cc.   All of these values were obtained during the late summer months; the mothers of the infants had been given a quart of milk and vitamin D in the form of cod liver oil daily for a period of approximately 1 month before delivery.   Jundell and Magnussen (24) observed a seasonal
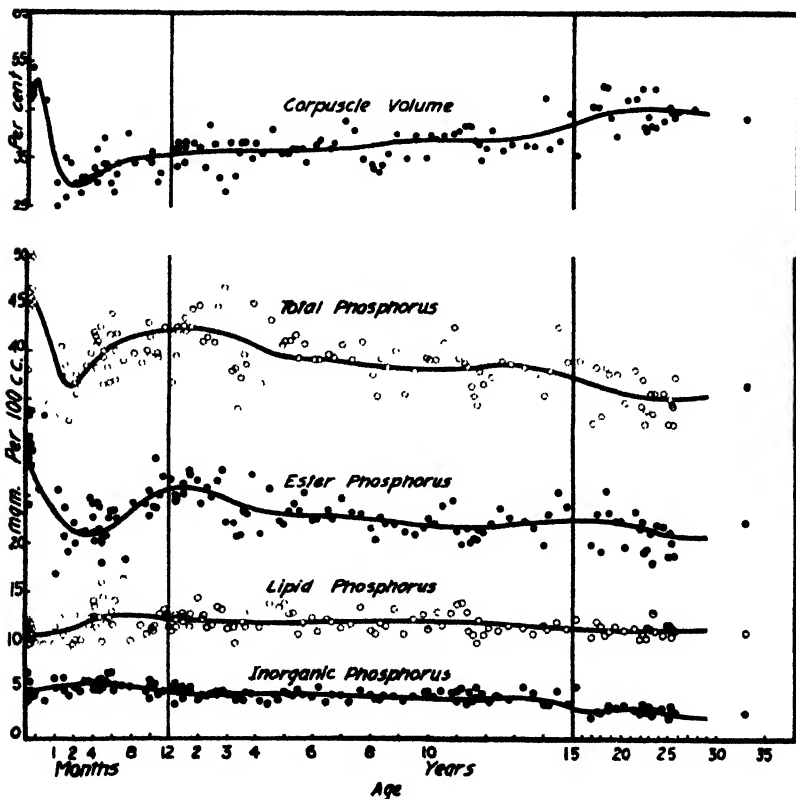
CHART 2. The corpuscle volume and partition of phosphorus in whole blood from birth to maturity.

variation in the inorganic phosphorus of cord serum, and also noted that the serum of infants whose mothers had been given additional vitamin D during pregnancy tended to show a higher inorganic phosphorus content than the serum of infants whose mothers had received no additional vitamins.   Our findings tend to corroborate their results.

The inorganic phosphorus of serum rises steadily after birth until the maximum value is reached at from 4 to 6 months of age. The height of the curve in infancy may be somewhat exaggerated in Chart 1, due to the fact that the majority of values in this group was obtained during the summer months. The values tend, in general, to confirm the findings of others (1, 3, 7-9, 26). The earlier workers (1, 7) reported higher values for breast-fed than for artificially fed infants. The values reported here for artificially fed infants are equal to those of the breast-fed infants studied by others. The increase is considered due to the increased intake of vitamin D by the artificially fed infants in the present study; vitamin D was not customarily given to infants in 1920.

From the 1st year through the 12th, the inorganic phosphorus of serum remains very nearly constant, the average value decreasing only from 5.5 to 5.0 mg. per 100 cc. These values compare well with those obtained by Bullock (8) from a study of New Orleans children, but are definitely greater than the average values of 3.9 mg. reported by Fedorova (28) for Russian children, and by Sokolovitch (15) for children in Glasgow.

Bullock (8) and Tisdall and Harris (29) noted an abrupt drop of serum inorganic phosphorus from the level of late childhood to the adult level. This drop occurred at about 19 years of age. In our series, this abrupt fall was observed somewhat earlier in the period of adolescence. The number of subjects observed during this period was too small, however, to determine with any accuracy the precise age at which this change in level occurs.

Lipoid P—The lipoid phosphorus of serum has been studied by several investigators. The low values observed in the new-born (5.3 mg. per 100 cc.) confirm the data reported by others (10–13, 30, 31). The level rises rapidly in early infancy, more slowly during later infancy, and reaches its maximum of 9.6 mg. at about 2 years of age. From then on, the amount decreases to an average value of 8.6 mg. by about the 4th year, a level which is maintained throughout childhood and early adolescence. The individual values for lipoid phosphorus show a much greater range (Chart 1) than was observed in the other phosphorus components.

Other investigators studying lipoid phosphorus of children (10, 11), have observed maximum values at somewhat different ages. Similarly, the average values reported for adults have varied

with different investigators (32–42). It seems probable that these variations may be ascribed largely to differences in diet, as György (10) has found that lecithin feeding definitely increased the serum lecithin of infants.

Ester P—The ester phosphorus (organic acid-soluble fraction) of serum is highest at birth, approximately 1 mg. per 100 cc. Similar values were observed by Plass and Tompkins (12) while McKellips, De Young, and Bloor (13) observed values averaging 2.2 mg. per 100 cc. in very young infants. As will be observed from Chart 1, after the 1st month of life, the ester phosphorus of serum remains practically constant throughout life. The small variations noted in the averages are within experimental error.

Total P—The total phosphorus of serum was determined as an analytical check against the analyses of the various fractions. The values obtained agree very well with those calculated from the sum of the lipid and acid-soluble fractions. The slope of the curve expresses the resultant of the other variables.

Serum Ca—High values for serum calcium were observed in the new-born (12.5 mg. per 100 cc.). Similar values have been noted by some investigators (43, 44) while others (45, 46) observed values closer to 11.0 mg. During infancy and early childhood the average value was 11.3 mg. per 100 cc. After the 3rd year of age, the serum calcium remained constant at 10.8 mg. per 100 cc. throughout the remainder of the period of growth. Kramer, Tisdall, and Howland (45) found the serum calcium constant through both infancy and childhood. The higher values recorded here for calcium as well as for the inorganic phosphorus of the serum in infants may be due, at least in part, to seasonal influence (47).

Plasma Phosphatase—Plasma phosphatase was determined whenever sufficient blood was available. The average value is low at birth, but rises abruptly to a maximum during the 1st month. This maximum level is maintained only a short time, though the average value remains at a comparatively high level until well into the 2nd year of life. The average level during middle childhood and adolescence is toward the upper limit of the normal adult range. The values found are in accord with the few values in infancy and childhood reported by others (48, 49).

The low values at birth were surprising. The phosphatase content of bone is highest just before birth (50). The phosphatase

of kidney and intestine, which apparently is identical with that of bone and plasma, is present in low concentration in the fetus but the content increases rapidly with beginning function of these organs.   If all of these tissues contribute to the plasma phosphatase, it would seem that the amount coming from bony tissue must be only about half of the total.   The reason for the drop in phosphatase after the 1st month of life is not clear.

Whole Blood

The values observed for phosphorus fractions in whole blood are dependent in large measure upon the quantity of corpuscles present in blood, because the largest fraction, the ester P, is carried almost entirely in the corpuscles.   It is necessary, therefore, to have some understanding of the changes which occur in the quantity of cells present in the blood stream at various ages.

According to Lucas and Washburn (51), the blood cell picture during infancy and childhood may be divided into three phases; that of the new-born, lasting from birth to the end of the 2nd or 3rd week; of infancy, which ends during the 2nd year of life; and of childhood.   The circulation of the new born infant is flooded with immature cells, and both red and white cells are increased in number.   The red cell count may reach 8 millions.   Before the end of the 1st month, the red cell count has fallen to between 4 and 5 millions, where it remains during the period of infancy.   The transition from infancy to childhood takes place between the 1st and 2nd year.   The changes in blood cells occur chiefly in the distribution of the various types of white cells.   From then on, no essential change in blood cell picture is noted from early childhood to adolescence, the red cell count averaging 5 millions throughout the entire period.

Corpuscle volume, rather than cell count, was studied in this series.   As oxalated blood was used, the volumes observed may be considered a little lower than the true values, but the results should be comparable.   The values are shown in Chart 2.   It will be observed that the corpuscle volume of the new born infants is higher than that of adults.   The corpuscle volume of capillary blood of infants under 3 weeks was also determined (Chart 3). An increase is noted during the first 3 days of life, which possibly may be ascribed to dehydration.   By the 8th day the drop in

corpuscle volume is distinctly noticeable. The quantity of corpuscles decreases steadily throughout the 2nd week. The lowest value observed was that of a 5 week-old infant whose corpuscles were only 25 per cent of the blood volume. The average value for six infants between 2 and 8 weeks of age was 30 per cent. After the low point is reached, the corpuscle volume increases slowly throughout infancy, reaching a value of about 36 per cent between 1 and 2 years of age. The value then increases very slowly throughout childhood, rising more sharply during early
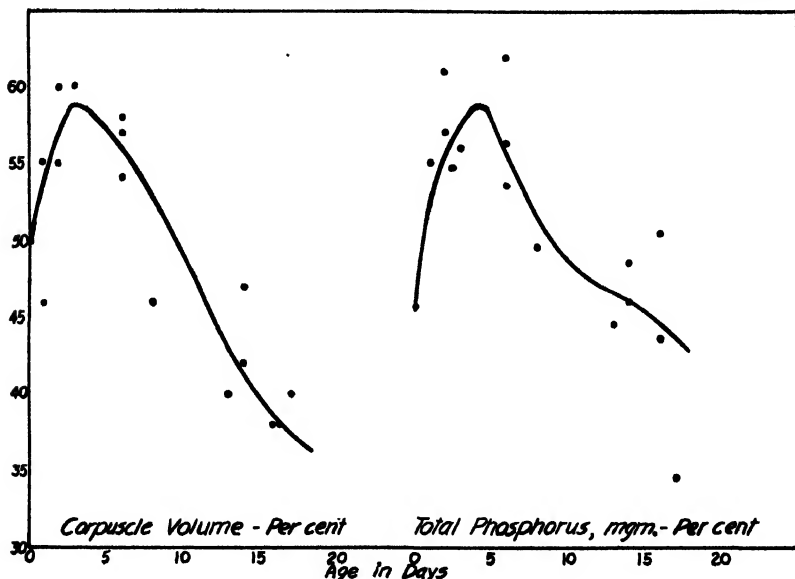
CHART 3. Corpuscle volume and total phosphorus of whole blood of infants from birth to 3 weeks of age.

adolescence. This latter increase may be due, in part, to the heavy predominance of males in the group. The average corpuscle volume in the adult group was 45.3 per cent, a value approximating those observed by others (33–36).

Inorganic and Lipoid P—No relationship was observed between the corpuscle volume and the quantity of inorganic or lipoid phosphorus in whole blood. The same changes as were noted in serum are observed in the whole blood values of these constituents, though to a less degree. The corpuscle values plotted in Chart 4

show a rise in lipoid phosphorus in corpuscles, similar to the rise in
serum lipoid phosphorus during infancy, with a later drop and a
comparatively constant value throughout childhood. The in-
organic phosphorus curve of corpuscles is almost a straight line
during infancy, dropping slightly at about the end of the 1st year,
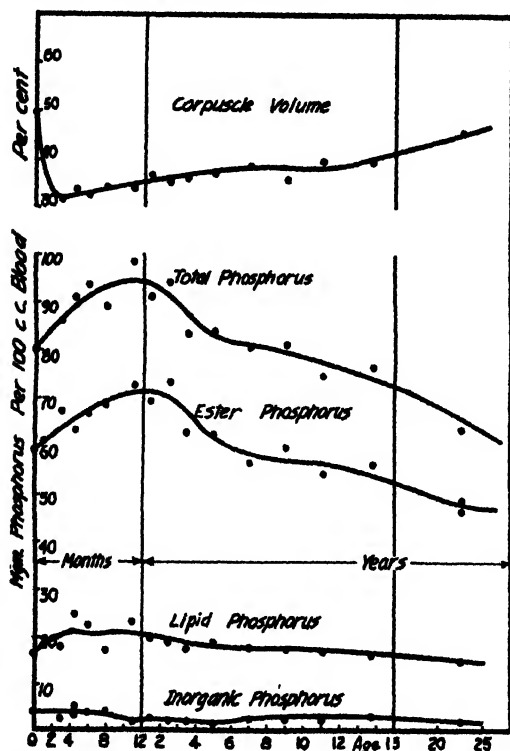
CHART 4. The partition of phosphorus in corpuscles from birth to
maturity.

then remaining constant throughout. The occurrence of inor-
ganic phosphorus in corpuscles has been questioned (52). If the
quantities found were due to the breakdown of organic phosphorus,
the amounts are very consistent.

Ester P—The values obtained for ester phosphorus in whole
blood of infants correspond well with those obtained by McKellips
and her collaborators for the same age group, but are somewhat

higher than the values obtained by other investigators (Table I). The values for children are at approximately the same level as those reported by Bomskov (17) and Sokolovitch (15), and higher than those given by Kay and Robison (16), and by Jones and Nye (14). The average value reported for adults is low, but within the general range reported by others (32–40).

The curve of ester phosphorus (Chart 2) tends to follow the curve of corpuscle volume during the period of infancy. After the 2nd year, however, the quantity of ester phosphorus in the whole blood falls slowly throughout childhood, whereas the corpuscle volume tends to rise. These differences become more marked during adolescence. The curve of ester phosphorus of corpuscles (Chart 4) shows that in the corpuscles this fraction rises steadily from birth, attaining a maximum during the 2nd year, then falls, reaching the birth level again at about the 6th year. From this time on the fall is more gradual, but constant, until the adult level is reached. The rise during the 1st month of life cannot be considered as compensatory to the fall in corpuscle volume because the rise then is no greater than that during the succeeding months, when the corpuscle volume is increasing.

These findings are not in accord with the conclusions reached by Byrom and Kay (53) after a study of the relation of ester phosphorus of whole blood to corpuscle volume in normal adults, and patients with secondary anemia or polycythemia. These authors concluded that the ester content of corpuscles is practically constant, and that therefore the factor, $\dfrac{\text{ester P}}{\text{corpuscle volume}}$, is a constant. The ester phosphorus of corpuscles in infants and children, on the contrary, changes continuously with the growth of the child.

The quantity of ester phosphorus in the blood of the individual child seems to depend somewhat upon the state of nutrition. Several of the normal children used as subjects were in the hospital for several weeks for another study. Their diet was of the "luxus" type. Even though the children were well, and up to normal weight at the beginning of the study, many of them showed improved color and other evidences of better nutrition after a month on the diet. The blood values obtained after several weeks stay in the hospital were from 1 to 3 mg. larger than those obtained upon entrance.

Total P—The total phosphorus values obtained by analysis agree excellently with those obtained by addition of the various fractions; therefore, the amount of other phosphorus compounds in blood not determined by this method must be negligible, even in the very young infants.   These results are contrary to the conclusions of Javillier and Fabrykant (40) who claimed to find 2.6 mg. of phosphorus as nucleoprotein phosphorus in normal adult blood.   The curve of total phosphorus in blood is of importance only as it represents the summation of the other components.

The total phosphorus of capillary blood was checked with that of venous blood and found to be practically identical.   Determinations were then made of total phosphorus and corpuscle volume of capillary blood of infants under 2 weeks of age (Chart 3).   The same degree of parallelism between the two determinations was observed as had been noted with the older infants.   The quantitative values obtained for phosphorus were in accord with those predicted from their age and the corpuscle volumes.   Because of the small amount of blood needed for total phosphorus determinations (0.1 cc. each for corpuscle volume and for phosphorus), which may be obtained without venipuncture, this determination may be of importance in the study of phosphorus, especially in very young infants.

Factors Influencing Phosphorus Levels.   Seasonal Variation

It did not prove possible to collect the data so as to give an equal seasonal distribution of observations in each group.   A study of such age groups as gave approximately the same number of analyses during the winter and summer months indicates that seasonal variation is probably at most only a minor factor in determining the quantity of ester phosphorus in blood.   The data, however, are by no means conclusive.   No seasonal variation was noted in the lipid fraction.

The general impression gained through the study of the individual children is that the state of nutrition has greater influence upon the organic phosphorus fraction of the blood than does the amount of sunlight.   The children in exuberant health showed the highest values, while children who were normal but probably not in optimal nutrition, gave values closer to the lower limits.

Effect of Continued Muscle Tension

The control group of young adults was composed chiefly of stutterers. These subjects were being studied for another purpose, and were expected to be normal in regard to phosphorus. Their extreme muscular tension at the time the blood samples were taken was so noticeable as to excite comment. Questioning elicited the fact that this extreme tension had been present for some time before the blood was taken. The values for inorganic and ester phosphorus were consistently low, either close to the low normal limit as reported by others, or even slightly below it. Similar findings were observed in child stutterers. Several of the group were tested a second time. Familiarity with the procedure caused a marked diminution of the nervous tension, the subjects were more relaxed, and the values obtained were definitely higher in all cases. The average serum inorganic phosphorus values of these subjects increased from 2.9 to 3.3 mg. per 100 cc.; the average ester phosphorus of whole blood from 19.4 to 23.4 mg. per 100 cc. Blood sugar values of the stutterers were determined simultaneously with the phosphorus analyses, but were always normal. Seven subjects who did not stutter, and who were known to be emotionally stable, were then studied. The values obtained for the phosphorus fractions agreed well with those obtained in the second determinations on the blood of the stutterers. Gemmill and Ribeiro (54) observed decreases in the inorganic and acid-soluble phosphorus of blood in two of three normal subjects 1 hour after severe muscular exercise. The amount of decrease was comparable to that noted in the group of stutterers. It would thus appear that the mental attitude of the subject, as reflected in the state of heightened muscle tonus, and fatigue for a period before the blood samples are taken, may affect temporarily the level of phosphorus in blood.

SUMMARY

1. The inorganic, lipoid, ester, and total phosphorus contents of whole blood and serum, together with the serum calcium and plasma phosphatase are reported in a series of 150 determinations on blood from 124 children ranging in age from birth to maturity.

2. No component of phosphorus studied is constant throughout the entire period of growth. Each component appears to reach a

maximum value at some time during infancy or early childhood. The age at which the maximum value is reached, and the length of time it is maintained vary for each component.

3. The curve of corpuscle volume during the period of growth was determined. The percentage of corpuscles in blood is high in the new-born but drops rapidly during the 1st month. The average value rises slowly during infancy and very slowly during childhood. The adult value is still somewhat lower than that at birth.

4. The serum calcium tends to be higher in the new-born than during later infancy. The level during infancy likewise tends to be higher than that during childhood. The values after the 3rd year remain constant throughout childhood and early adult life.

5. The phosphatase of plasma is low at birth but rises very rapidly to a maximum during the 1st month of life and remains fairly high until during the 2nd year. The values in later childhood are within the adult range.

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THE ELECTROMETRIC TITRATION OF CREATINE ESTER HYDROCHLORIDE AND SOME RELATED COMPOUNDS*

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The dissociation constants of creatine and creatinine have been determined by a number of observers, the most recent measurements being those of Cannan and Shore (1) who found for creatine pK = 2.66 and for creatinine pK = 4.78, both at 25°. It was thought interesting to investigate creatine methyl ester hydrochloride, isocreatine (N-methyl-N′-carboxymethylguanidine), guanidoacetic acid, its anhydride glycocyamidine, and the methyl ester hydrochloride of guanidoacetic acid.[1]

EXPERIMENTAL

Measurements of pH were made with the Clark rocking electrode at a temperature of 25° and referred to 0.1 N hydrochloric acid, pH 1.08. The hydrogen used was passed over copper at a dull red heat. All organic compounds were present at the concentration 0.02 M, each solution being made up separately to volume after the appropriate addition of acid or alkali.

Titration curves for creatine, isocreatine, and guanidoacetic acid are plotted in Fig. 1 with pH as abscissa and mols of added hydrochloric acid per mol of substance as ordinate. It may be seen that in acid solution the points for isocreatine and guanido-

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[1] This last compound, which is not described in the literature, was prepared by esterification of guanidoacetic acid. Its general properties together with some observations on the chemical behavior of certain guanido compounds will be reported elsewhere (2).

acetic acid coincide but differ from those for creatine. In alkaline solution all three titration curves coincide with the water blank. The pK values[2] found are isocreatine 2.88, guanidoacetic acid 2.86, creatine 2.67. Thus creatine has a pK lower by 0.20 unit than have the related compounds containing 1 hydrogen and 1 carbon atom attached to the nitrogen on the α-carbon, instead of 2 carbon atoms as in the case of creatine. This corresponds closely to the difference for methylglycine pK 2.15 and dimethylglycine pK 1.93, which amounts to 0.22 unit (4).

Creatine methyl ester hydrochloride was titrated with sodium hydroxide. No dissociation constant could be calculated, as an
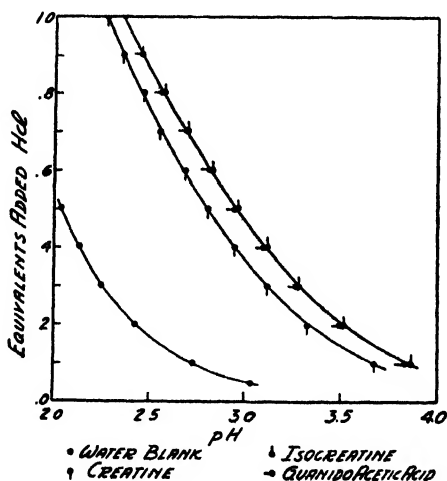
Fig. 1. Titration curves of creatine, isocreatine, and guanidoacetic acid

irreversible change to creatinine takes place.[3] On adding hydrochloric acid to the alkaline solution, however, a back titration curve was obtained, differing from that of the ester hydrochloride

[2] pK is defined (3) as $-\log (Kw/Kb)$.

[3] Ester salts of creatine were first prepared by Dox and Yoder (5). Contrary to Kapfhammer (6) and to Hunter (7), these compounds are regarded by Schotte, Priewe, and Roescheisen (8), Hynd and Macfarlane (9), Brand and Harris (10) as derivatives of creatine and not of creatinine, notwithstanding their rapid transformation into creatinine upon neutralization. The free esters of certain guanido acids are apparently quite unstable (cf. (11, 12)).

and giving a pK value of 4.83 which agrees with the value 4.84 found by the authors for creatinine. In Fig. 2 is shown the course
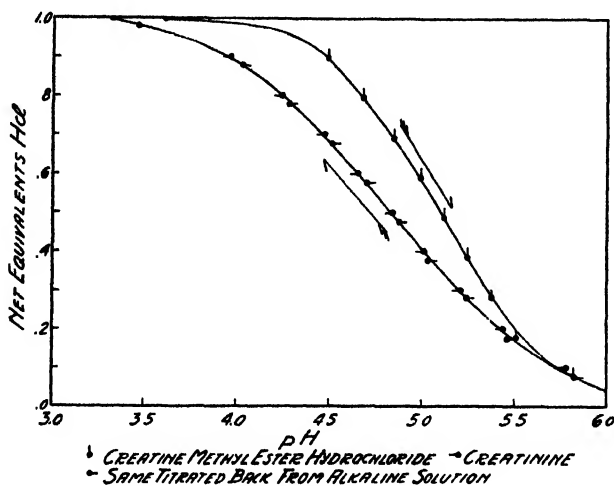
FIG. 2. Titration curves of creatine methyl ester hydrochloride and creatinine.
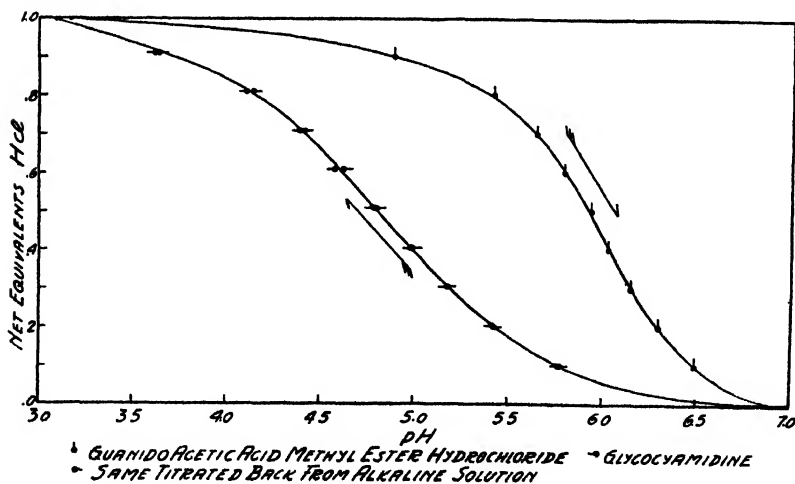
FIG. 3. Titration curves of guanidoacetic acid methyl ester hydrochloride and glycocyamidine.

of the titration, with arrows indicating the sense in which the curve is being traversed. Points obtained on adding acid to an

alkaline solution of the ester are seen to coincide with the curve representing the addition of acid to creatinine.

A similar titration with guanidoacetic acid methyl ester hydrochloride is plotted in Fig. 3, together with points on the curve for glycocyamidine, pK 4.80, and the back titration of the guanidoacetic acid methyl ester from alkaline solution, pK 4.81. In the most acid solutions the pK values calculated for these two substances become slightly lower, perhaps due to an equilibrium being established between glycocyamidine and guanidoacetic acid.

DISCUSSION

These experiments furnish additional evidence that in alkaline solution the methyl esters of creatine and of guanidoacetic acid are transformed into the corresponding anhydrides. The possibility suggests itself that in the usual estimation of creatinine in alkaline solution there may appear as creatinine any unstable creatine compounds present, together with that creatinine which already exists as such (10). The possible physiological importance of such mechanisms for creatinine formation has also been pointed out previously (13).

Titration curves for creatinephosphoric acid before and after splitting into its components were obtained by Meyerhof and Lohmann (14). While creatinephosphoric acid is more acid before splitting (by enzymatic or acid hydrolysis), creatine ester is more basic before anhydride formation induced by the spontaneous splitting off of alcohol in a neutral or alkaline medium.

SUMMARY

1. The following dissociation constants were determined at 25°: creatine, 2.67, isocreatine (N-methyl-N′-carboxymethylguanidine) 2.88, guanidoacetic acid 2.86, creatinine 4.84, glycocyamidine 4.80.

2. The hydrochlorides of creatine and of guanidoacetic acid methyl esters were titrated with NaOH. No dissociation constants could be calculated, as an irreversible change to the anhydrides takes place. On adding HCl to the alkaline solution, a back titration curve was obtained differing from that of the ester salts and giving a pK value which agreed with the values found for creatinine and glycocyamidine respectively.

3. The possible physiological aspects of the behavior of creatine esters were discussed.

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THE RELATION OF THYROID TO THE CONVERSION OF CYANIDES TO THIOCYANATE*

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In 1932 Marine, Baumann, Spence, and Cipra (1) discovered that administration of cyanides to rabbits will usually lead to thyroid hyperplasia. As one phase of our study of the mechanism of this phenomenon we have investigated the fate of several cyanides given to normal and thyroidectomized rabbits with the results presented in this paper.

Lang (2) first showed that 12 to 17 per cent of administered cyanide (KCN and CH_3CN) is detoxicated by conversion to thiocyanate and can be recovered from the urine as such. Hunt (3) also recovered considerable amounts of thiocyanate from the urine of guinea pigs injected with acetonitrile. Unfortunately his animals were fed on a diet containing cabbage, and we now know that the *Cruciferæ* contain large amounts of thiocyanate. Since this early work, the Rupp and Schied (4) method for estimating SCN has been chiefly used. While capable of yielding satisfactory determinations on pure solutions, we have found that this method gives results from 35 to 1000 per cent too high, when applied to urine and other animal matter.

In the work reported here, the thiocyanate excretion of rabbits is used as a measure of cyanide metabolism and is determined by oxidation to HCN and estimation of the latter by the Liebig-Déniges method. With the amounts measured in these experiments, results correct within about 5 per cent are readily obtained. The

rabbits were fed 35 gm. each of alfalfa hay and oats daily. Excretion of SCN on this diet is about 0.3 to 0.4 mg. per day. Urines were collected usually in 2 day periods. The bladders were not compressed, so that sharp 48 hour collections were not obtained.

KSCN—Control experiments were made to determine the amount of thiocyanate that could be recovered from the urine of rabbits after injecting definite amounts of KSCN subcutaneously.

TABLE I

Excretion of Thiocyanate after Subcutaneous Injections of KSCN

Rabbit No. and sex.	1114 ♀	1117 ♀	1118 ♀	1159 ♂	1168 ♂	1184 ♂	1209 ♂
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1. Total KSCN injected in 3 days, calculated as SCN	18.27	18.27	77.88	84.75	84.75	20 43	77.88
2. Total SCN found in urine in 5 days.......	18 86	19 99	78 97	71.71	78.73	20 31	66.10
3. Total SCN normally excreted in urine in 5 days on standard ration......	2.45	2 45	2.10	2 25	2.33	1.88	1.70
4. Amount of injected SCN recovered in urine ((2) − (3)). .	16.41	17.54	76 87	69.96	76.40	18.43	64.40
5. Per cent of injected SCN recovered in urine..........	(90)*	(96)	(99)	(83)	(91)	(90)	(83)

* The figures in parentheses are measured in per cent.

Animals were injected with 1 cc. of standardized potassium thiocyanate solution from a calibrated tuberculin syringe on 3 successive days.

The data are summarized in Table I. In the case of two male rabbits, we believe the comparatively low recoveries are due to technical difficulties. They were especially active and scattered their urine all over the cage, making quantitative washing of the cages very difficult if the volume of washings was to be kept reasonably low. With animals satisfactory for metabolism experiments, at least 90 per cent can be recovered. One may conclude,

therefore, that once cyanide has been converted to SCN nearly all will be found in the urine within 24 hours. These results are similar to those of Pollak (5) with dogs, man, and rabbit, and of Smith and Malcolm (6) with dogs.

CH_3CN—The influence of acetonitrile injections on the SCN excretion of thyroidectomized rabbits was studied for 2 months.[1]

TABLE II

Excretion of Thiocyanate after Subcutaneous Injection of Acetonitrile in Thyroidectomized Male Rabbits

	Average SCN excretion per day				Remarks
	Rabbit 1051	Rabbit 1065	Rabbit 1072	Rabbit 1082	
	mg.	*mg.*	*mg*	*mg.*	
Mar. 18–24.......	4 47	6.75	7.92	4.98	0.05 cc. CH_3CN daily
" 25–31.......	3.48	5.41	5.27	4 85	
Apr. 1– 8.......	4.31	5.79	4 35	3.98	
" 9–18.......	5 82	6.70	9 01	4 16	Dose increased to 0.1 cc.
	(5.2)*	(5.9)	(8.0)	(3 7)	Per cent of injected CH_3CN recovered as SCN
" 19–26....	9 73	10 59	21 21	10 17	Rabbits 1051 and 1065, 4 doses 25 mg. desiccated thyroid on alternate days Rabbits 1072 and 1082, 4 doses 100 mg. desiccated thyroid on alternate days
Apr. 27–May 1 .	7.92	10 28	10.96	7 73	Rabbit 1072, only 1 dose 0.1 cc. CH_4CN in these 5 days
May 2– 8. ..	7.25	6.75	0 51	6 39	Rabbit 1072, no CH_3CN
" 9–13	11.66	7 36	18.80	7 27	" 1072, CH_3CN injections resumed
" 14–17.....			15 08	5.28	

* The figures in parentheses are measured in per cent.

3 days after operation, daily intramuscular injections of 0.25 cc. of a 20 per cent (by volume) acetonitrile solution were started. After 3 weeks the daily dose was increased to 0.5 cc. of the 20 per cent solution, at which level it was continued to the end of the experiment. The data are summarized in Table II.

[1] We have been unable to detect even 0.05 mg. of SCN in 20 gm. of rabbit feces.

We have found that rabbits with normal thyroids excrete about 30 to 40 mg. of SCN per day when injected with 0.1 cc. (0.08 gm.) of acetonitrile; i.e., about 27 to 35 per cent appears in the urine as SCN. This compares with the 12 to 17 per cent found by Lang in the urine of a dog fed rather larger doses of CH_3CN mixed with the food.

During the first 5 days of acetonitrile injection (3rd to 8th day after thyroidectomy) while there is still considerable circulating thyroid hormone in the rabbits, and while the tissues are reaching the level of acetonitrile concentration that results from a daily dose of 0.05 cc., the SCN excretion gradually increased from 3 to 8 or 10 mg. Then the amount falls to the thyroidectomy level which is 3 to 4 mg. for Rabbits 1051 and 1082 and 5 to 6 mg for Rabbits 1065 and 1072. At autopsy fragments of regenerating thyroid tissue were found in the latter two animals while no thyroid was found in the first two. In other words the SCN excretion varies directly with the metabolic rate.

Doubling the daily dose of acetonitrile caused only a slight increase in the absolute SCN excretion in three of the four rabbits so that it still equaled only 3 to 5 per cent of the amount theoretically possible. In Rabbit 1072 in which the larger thyroid stump remained, the amount of SCN excreted doubled.

We next studied the effect on SCN excretion of feeding desiccated thyroid to these thyroidectomized rabbits. With 100 mg. of thyroid the SCN excretion increased 60 and 100 per cent, while with 400 mg. the SCN excretion at its height was tripled. The rise began within 24 hours, continued during thyroid administration, and decreased slowly when thyroid feeding was stopped, though 3 weeks after some action of thyroid was still evident. This continued activity is similar to the influence of thyroid on total metabolism.[2]

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[2] The behavior of Rabbit 1072, the one in which most thyroid tissue was found at autopsy, is of special interest. It received the last dose of 100 mg. of thyroid on April 25. It did not eat its food that day. About 5 hours after the injection of acetonitrile on April 26 it showed slight symptoms of cyanide intoxication from which it had recovered by the morning of April 27. Injection of the regular 0.10 cc. of acetonitrile brought forth in 4 hours a much more marked intoxication, from which it recovered to a large extent the following morning. Injections of acetonitrile were discontinued for 10 days. The SCN excretion which had been around 8 mg. per day be-

From these observations it is clear that without thyroid tissue the SCN excretion of rabbits injected with acetonitrile is reduced to about 15 per cent of the normal, and that when fed with thyroid the SCN excreted increases, approaching that of the normal animal, if the amount of thyroid given is sufficiently great. Thyroid therefore is necessary for the efficient conversion of the CN of acetonitrile to SCN.

These findings are in harmony with those of Schechter (7) that injections of both acetonitrile and thyroid increase the SCN content of guinea pig serum, but not with his observation that thyroidectomy has no influence on the serum SCN content of guinea pigs similarly injected with acetonitrile.

The accelerating influence of thyroid on the catabolism of CH_3CN might be due to a more intense demethylation, a more rapid conversion of CN to SCN, or to both these processes.

$$CH_3CN \rightarrow {}^-CN \rightarrow {}^-SCN$$
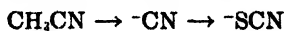
KCN—By studying the formation of SCN from KCN, the process of demethylation could be eliminated and the effect of thyroid on the detoxication of CN could be examined by itself. To this end, four rabbits were injected subcutaneously twice each day with 1 cc. of KCN solution, containing 1 mg. of CN, for 9 days, after which they were thyroidectomized. The SCN output in the urine was determined for 21 days as before. The data are summarized in Table III.

fore beginning thyroid feeding increased to 26 mg. When severe symptoms of cyanide poisoning appeared the SCN excretion dropped to 7 mg. per day for 2 days, after which it averaged 13 mg. daily for 3 days *although no acetonitrile was administered during this time.* Once symptoms of cyanide intoxication are present the ability of the body to detoxicate that cyanide seems to be greatly diminished, even when, as in this case, an adequate amount of thyroid is present. We have made similar observations in other rabbits. When injections of acetonitrile were resumed, the SCN excretion again increased considerably but not to as high a level as immediately after the period of thyroid medication. On May 17, the rabbit ate only part of its ration and on the following day it again showed severe symptoms of cyanide poisoning from which it died.

The increased sensitivity of this rabbit to acetonitrile after thyroid medication is in line with Hunt's observation but contrasts with the behavior of mice, which, he found, become more resistant to the action of acenitrile on feeding with thyroid.

Unlike our experience with CH_3CN, nearly all the potassium cyanide is converted to thiocyanate and is excreted within 24 hours. We recovered as SCN 79 and 83 per cent of the amount of CN in-

TABLE III

Excretion of Thiocyanate after Injections of Potassium Cyanide in Normal and Thyroidectomized Rabbits

Rabbit No. and sex.	1180 ♀	1181 ♀	1182 ♂	1183 ♂
Before thyroidectomy				
	mg.	*mg.*	*mg.*	*mg.*
Average KCN injected Sept. 22–30 equivalent to SCN per day.	4.53	4.55	4.53	4.53
Average urinary output SCN per day, Sept. 22–30	4 13	4.21	3 32	3.59
Average urinary output SCN per day on standard ration.	0.53	0.46	0.51	0.45
SCN derived from injected KCN per day	3 58	3 75	2.81	3.14
Per cent of KCN injected, recovered as SCN..	(79)*	(83)	(62)	(69)
After thyroidectomy				
Average KCN injected per day equivalent to SCN.	4.46	4 46	4.46	4.46
Average SCN excretion per day, 1 to 5 days after thyroidectomy	3.87	3.61	3.34	3.58
Average SCN excretion per day, 6 to 10 days after thyroidectomy	2.87	2.17	2.25	2.91
Average SCN excretion per day, 15 to 18 days after thyroidectomy.......	4.24	3.43	3.52	3.63
Average SCN excretion per day on standard ration....	0.53	0.47	0.50	0.40
SCN derived from injected KCN per day for period 15 to 18 days after thyroidectomy...	3.71	2.96	3.02	3.23
Per cent of KCN injected recovered as SCN for period 15 to 18 days after thyroidectomy.....	(81)	(66)	(68)	(72)

* The figures in parentheses are measured in per cent.

jected in the case of the females and 62 and 69 per cent from the urine of the males. These figures are comparable to those of Smith and Malcolm (6) in experiments on dogs.

After thyroidectomy the SCN output decreases only slightly during the first 5 days, but in the next 5 days a drop to 50 to 65 per cent of the preoperative level is reached, after which it gradually rises again so that during the 15 to 18th day after operation, the former normal level is reached.   At autopsy small hyperplastic regenerating fragments of thyroid were found in three of the four rabbits.   So that while thyroidectomy has a definite effect in

TABLE IV

Influence of Thyroidectomy on Excretion of Thiocyanate after Subcutaneous Injections of Benzyl Cyanide

Rabbit No. and sex.	1114 ♀	1117 ♀	1168 ♂
	mg.	*mg*	*mg*
BzCN injection per day equivalent to SCN.	12.50	12 50	12 50
Average normal SCN excretion per day...	0 54	0.53	0 50
" daily SCN excretion due to BzCN before thyroidectomy (5 days)	8.71	8.45	6.35
Per cent of injected BzCN recovered as SCN before thyroidectomy..	(70)*	(68)	(51)
Average daily SCN excretion due to BzCN 1 to 5 days after thyroidectomy .	8 73	9 03	8 86
Average daily SCN excretion due to BzCN 6 to 12 days after thyroidectomy. .	7 84	8 25	8.06
Per cent of injected BzCN recovered as SCN 6 to 12 days after thyroidectomy	(63)	(66)	(64)
Average daily SCN excretion due to BzCN 13 to 19 days after thyroidectomy .	8 59	8 49	4.42
Average daily SCN excretion due to BzCN 20 to 25 days after thyroidectomy .	9.24	8.91	4.40
Per cent of injected BzCN recovered as SCN 20 to 25 days after thyroidectomy	(74)	(71)	(35)

* The figures in parentheses are measured in per cent.

lowering the SCN output, the influence is only a temporary one. We conclude, therefore, (1) that thyroid is *not* necessary for the conversion of ionized cyanide to thiocyanate; (2) since the thyroidectomized rabbit can convert only 3 to 5 per cent of injected acetonitrile to thiocyanate, while the normal rabbit converts 27 to 35 per cent of similar amounts of acetonitrile, we further conclude that thyroid increases the ability of the body to demethylate acetonitrile 7-fold.

BzCN—Finally to discover whether thyroid aids in the cleavage of other nitriles or whether its action is specific for acetonitrile, we studied the fate of benzyl cyanide injected into rabbits before and after thyroidectomy. The results of this experiment are summarized in Table IV. Three rabbits were injected with 1 cc. of a 2.5 per cent emulsion of benzyl cyanide daily, equivalent to 12.5 mg. of SCN. The average daily SCN excretion amounted to approximately 70 per cent of the cyanide administered in the female and 50 per cent in the male.

The rabbits were thyroidectomized and daily injections of benzyl cyanide continued. In the female rabbits, no significant change in the SCN excretion occurred during the first 5 days after thyroidectomy. In the next 7 days a slight decrease was observed, not, however, outside the limits of error of these experiments, after which there was a return to the preoperative level. In the case of the male rabbit, the SCN excretion (which before thyroidectomy had started at over 8 mg. per day but decreased significantly by the 4th and 5th days) increased for the first 12 days after thyroidectomy to over 8 mg. per day. For the following 2 weeks the daily excretion averaged only 4.4 mg. We are not inclined to regard the decrease in this instance as having anything to do with a lack of thyroid but rather as a peculiarity connected with the sex of this animal. The difference between male and female is similar to though greater than that observed in the SCN excretion of rabbits after administering acetonitrile, potassium cyanide, and even potassium thiocyanate. In each case less SCN was excreted by the male than by the female rabbits. Moreover, on autopsy a small fragment, 2 × 3 mm., of regenerating thyroid was found in this male which on histological examination proved to be markedly hyperplastic, while postmortem examination revealed no thyroid in the two female rabbits. Thyroidectomy had little or no influence on the capacity of rabbits to convert benzyl cyanide to SCN.

DISCUSSION

These experiments show that in the absence of thyroid only 3 to 5 per cent of the cyanide of administered acetonitrile is converted to thiocyanate; whereas with the thyroid intact 27 to 35 per cent of the cyanide is transformed. This catalyzing influence of thy-

roid is specific for acetonitrile. The conversion of the CN radicals of potassium cyanide or of benzyl cyanide to thiocyanate is little affected by the presence or absence of thyroid, and is nearly complete. In other words, thyroid catalyzes the demethylation of acetonitrile. Demethylation of many compounds is brought about only with considerable difficulty by the mammalian organism. For example, the methylated purines are demethylated slowly and incompletely. Creatinine is almost entirely excreted without further oxidation. It would be interesting to know whether thyroid aids in demethylations that do take place in the body.

On the other hand, Stuber *et al.* (8) have ascribed a specific methylating function to the thyroid. They find that guanidineacetic acid is readily methylated in the normal rabbit but not after the thyroid has been removed. Most reactions that have been associated with the thyroid are catabolic in nature—and demethylation would of course belong to this class. It must be admitted, however, that very little is known of the specific reactions that thyroid hormone initiates or accelerates, the methylation of guanidineacetic acid and the demethylation of acetonitrile being among the first.

We find no evidence for the detoxifying action Schechter (7, 9) has ascribed to the thyroid in forming SCN from CN.

SUMMARY

The relation of thyroid to the formation of thiocyanate from methyl cyanide, benzyl cyanide, and potassium cyanide is described. In normal rabbits 27 to 35 per cent of the CN radical of methyl cyanide is converted to SCN, whereas in thyroidectomized rabbits only 3 to 5 per cent is so changed. With benzyl cyanide or potassium cyanide about 75 per cent of the amounts given are converted to thiocyanate. After thyroidectomy there is only a small and temporary decrease in the amount excreted, returning within 2 weeks or so to the preoperative level. When inorganic thiocyanates were injected 90 per cent could be found in the urine. The recoveries with potassium cyanide and benzyl cyanide are therefore nearly complete and are not influenced by thyroid. We conclude that the thyroid catalyzes demethyla-

tion of acetonitrile but has nothing to do with the formation of thiocyanate.

We are indebted to Dr. David Marine for performing the operations and for making the anatomical and histological examinations.

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Entries are not made for physical constants or properties, but only under the substance in question. Thus, the *Absorption curve of carbon dioxide* will be found only under *Carbon dioxide; Optical rotation of glucose*, only under *Glucose*.

Similarly, physiological phenomena such as *Absorption, Assimilation, Digestion, Equilibrium, Excretion, Fermentation, Metabolism, Respiration*, etc., are not indexed as such, but only under the substance in question.

When a definite constituent is given in connection with *Biological fluids, Biological material, Blood, Blood cell, Blood plasma, Blood serum, Diet, Milk, Tissues, Urine*, the constituent only is indexed; *i.e., Cholesterol in blood*, etc., is indexed under *Cholesterol*, not under *Blood*, etc.   However, *Blood sugar* is indexed as such.

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